力な肺転移抑制効果を得ることができた。このことから、バブルリポソームと超音波の併用が外科的療法後のがん転移予防における有用な抗原送達システムになることが示唆された。

F. 健康危険情報

該当無し

G. 研究発表

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H. 知的財産権の出願・登録状況

1. 特許取得
   該当無し
2. 実用新案登録
   該当無し
3. その他
   該当無し

厚生労働科学研究費補助金（第3次対がん総合戦略研究事業）
分担研究報告書

低栄養・低酸素環境により誘導される抗がん剤耐性機構

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固形がん組織の微小環境を模倣する低栄養・低酸素環境により誘導される膵癌細胞のゲムシタビン耐性には薬剤のゲノム DNA 取り込み後の細胞死誘導機構の抑制が関与していることが示された。2種類のキナーゼ阻害剤の併用によりこの耐性が部分的に解除されることが明らかとなった。

A. 研究目的

膵癌をはじめとする固形がん組織では血流低下に起因する低栄養・低酸素環境の形成が想定される。これまでがん細胞のこうした微小環境への適応を明らかにし、低栄養環境選択的に細胞毒性を発揮する化合物（栄養飢餓耐性解除薬）の探索など、この適応反応を標的とした治療法の開発を進めてきた。さらに低栄養・低酸素環境が既存の抗がん剤に対する耐性を誘導することを見出し、それが従来言われてきた耐性機構とは異なる要因によることを示し、さらにこの耐性を解除する分子標的療法の可能性について検討した。

B. 研究方法

1）　ゲムシタビンによる細胞死が顕著に抑制される低栄養・低酸素培養条件下での DNA 修復関連タンパク質の活性化およびカスパーゼの酵素活性を検討した。

2）　キナーゼ阻害剤 LY294002 および UCN-01 の単剤及び併用による低栄養・低酸素に誘導されるゲムシタビン耐性の解除を検討した。

3）　低酸素に誘導される転写開始点の高精度網羅的解析を次世代 DNA シーケンサーにより行った。

C. 研究結果

1）　通常条件での膵癌細胞株 PANC-1 に対するゲムシタビンの IC_{50} は 300 nM であったが、無グルコースあるいは通常の 1/10 のグルコース濃度かつ低酸素(1%)条件では細胞毒性が認められなかった。ただし DNA へのゲムシタビンの取り込みは減少せず、DNA 損傷修復に関わる H2AX, Chk1,2 のリン酸化も通常条件と同様に観察され

た。一方、通常条件でゲムシタビン投与により上昇したカスパーゼ活性は低栄養・低酸素条件では顕著に減弱しており、DNA 損傷後のアポトーシス経路の阻害が耐性機序とかかわることが示唆された。

2） PI3K の特異的阻害剤 LY294002 および PKC, Chk1 等を阻害する UCN-01 をそれぞれ単独でゲムシタビンと併用したところ、通常条件ではゲムシタビンの毒性を増強したが、低栄養・低酸素条件で誘導される耐性は解除されなかった。しかし LY294002 と UCN-01 を同時に添加すると、低栄養・低酸素条件におけるゲムシタビン耐性が一部解除された。このとき、抑制されていたカスパーゼ活性も回復していた。

3） 大腸癌細胞株 DLD-1 をモデルに低酸素に応答する 120 個の新規および既知のタンパク質コード遺伝子、220 個の新規 non-coding gene を同定した。

D. 考察

膵癌の標準治療薬であるゲムシタビンの効果が腫瘍組織の微小環境を模倣した低栄養・低酸素条件下で著しく減弱することから、生体内においてゲムシタビンの効果が十分発揮されない可能性が危惧された。低栄養・低酸素に誘導されるゲムシタビン耐性は従来言われてきた耐性獲得機序とは異なっていたが、これが LY294002 と UCN-01 の併用で初めて解除され、耐性機構には PI3K とそれ以外の細胞内シグナル伝達機構が同時に関与していると想定された。さらに詳細に責任分子を同定し今後これを標的としたゲムシタビン併用療法の開発につなげたい。さらに、低栄養環境下で殺細胞作用を誘導する分子機構は、これまで同定してきた一連の栄養飢餓耐性解除薬の作用機序と重複する可能性もあり、今後検討する。

E.結論

本研究により、腫瘍組織で見られる低栄養・低酸素環境に誘導される代謝拮抗薬に対する耐性が複数の分子標的薬の併用で解除される可能性が示された。

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分担研究報告書

微生物代謝産物からの抗がん剤の開発に関する研究

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プロジェクト研究推進ユニット長

研究要旨　がん治療薬の開発を目的として、2つのスクリーニング系を用いてリード化合物を探索した。固形がん内部に見られる栄養欠乏状態の細胞に選択的に細胞毒性を示す化合物として、Penicillic acidおよびPapyracillic acidを見いだした。これらは培地中のアミノ酸の欠乏環境において強い細胞毒性を示した。また放線菌の生産するプロテアソーム阻害剤チロペプチンの誘導体が、がん移植モデルマウスにおいて強い抗腫瘍活性を示した。

A.　研究目的

がん治療薬の開発を目的として、2つのスクリーニング系を用いてリード化合物の探索を行った。1つ目は固形がん内部に見られる慢性的な栄養飢餓状態のがん細胞に選択的に細胞毒性を示す化合物を微生物代謝産物よりランダムスクリーニングした。2つ目はがん細胞の増殖に重要なタンパク質分解酵素であるプロテアソームをがん治療の分子標的として定め、放線菌の生産するプロテアソーム阻害剤チロペプチンの誘導体を合成した。

B.　研究方法

栄養飢餓選択的細胞毒性物質の探索には、ヒト膵がん PANC-1 細胞を用い、栄養培地として DMEM(10% FBS 含)、栄養飢餓培地として NDM (DMEM からグルコース、アミノ酸を除いた培地に透析した FBS を 10%含む)を使用し、DMEM (10% FBS)培地に較べ NDM(10% D-FBS)培地で選択的に細胞毒性を示す物質を放線菌およびカビの培養液より探索した。微生物培養液からの活性物質の単離精製には、溶媒抽出法、各種カラムクロマトグラフィー法、HPLC 等を適宜組み合わせることにより行なった。単離した化合物は TLC 呈色反応、NMR、MS、IR、UV スペクトルの詳細な解析により、それらの化学構造を決定した。プロテアソーム阻害剤チロペプチン誘導体は、所属機関の別ユニット

で合成を行い、biomol 社の 20S プロテアソームを用いて阻害活性を測定した。In vivo 抗腫瘍活性の測定は、scid マウスにヒト多発性骨髄腫 RRPMI8226 細胞を鼠径部皮下に移植したヒトがんモデルマウスを用いて治療実験を行った。

（倫理面への配慮）

当研究センター内の動物実験指針および環境安全委員会規定に従い実験を行なった。

C. 研究結果

数千の微生物培養液より栄養飢餓選択的細胞毒性物質のランダムスクリーニングを行ったところ、2種のカビ固体培養物の抽出液に強い栄養飢餓選択的細胞毒性を認めた。各玄米400g分のカビ固体培養のアセトン抽出液より各種クロマトグラフィーを組み合わせて活性成分を単離精製したところ、それぞれ既知低分子化合物であるPenicillic acidおよびPapyracillic acidであった。これらの化合物は栄養培地DMEM（10% FBS）に較べて栄養飢餓培地NDM(10% D-FBS)で強い細胞毒性を示した。この栄養飢餓選択的細胞毒性に影響を与える培地成分について調べたところ、グルコースや血清の欠乏では栄養培地と同程度の細胞毒性のままであったが、アミノ酸を欠乏すると強い細胞毒性を示した。

また放線菌の生産するプロテアソーム阻害剤チロペプチンの新規誘導体を合成したところ、いくつかの誘導体がプロテアソームのキモトリプシン様活性を強く阻害することがわかった。これらはヒト多発性骨髄腫RPMI8226細胞やヒト膵がんPANC-1細胞に強い細胞毒性（数nMのIC_{50}値）を示し、免疫不全Scidマウスの鼠径部皮下にヒトがんRPMI8226細胞を移植したヒトがんモデルマウスを用いてがん治療実験を行ったところ、4mg/kgを週に2回静脈内投与において明らかな腫瘍増殖抑制効果を認めた。

D. 考察

栄養飢餓選択的細胞毒性物質として Penicillic acid および Papyracillic acid を見い出すことができた。本化合物はグルコースおよび血清の有無に関わらずアミノ酸欠乏でのみ強い細胞毒性を示し、これまでにないユニークな作用の化合物であった。これら化合物は代謝回路に作用する可能性があり、その作用機序の解明はがん治療の新たな標的分子の発見およびがん微小環境における代謝の役割の解明に繋がる可能性が考えられた。また今回合成した新規プロテアソーム阻害剤チロペプチン誘導体が、in vivo で抗腫瘍活性を示したこ

とは、これら誘導体が抗がん剤になりうる可能性が示唆されたと共に、がん治療の分子標的としてプロテアソームは有望であることがわかった。

E. 結論

本研究において、栄養飢餓選択的細胞毒性物質としてPenicillic acidおよびPapyracillic acidを見い出し、これら化合物がアミノ酸欠乏において選択的に細胞毒性を示すことがわかった。また新規プロテアソーム阻害剤チロペプチン誘導体が抗腫瘍活性を示し、がん治療への応用が示唆された。

F. 健康危険情報

該当なし

G. 研究発表

1. 論文発表

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プロテアソーム分解性蛍光タンパク質を用いた腫瘍内プロテアソーム阻害活性の in vivo イメージング:百瀬 功、立田大輔、大庭俊一、増田 徹、池田大四郎

2) 第68回日本癌学会学術総会

In vivo imaging of proteasome inhibitory activity in tumor of living mice. :百瀬 功、増田 徹、立田大輔、池田大四郎

H. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

厚生科学研究費補助金（第３次対がん総合戦略研究事業）
分担研究報告書

腫瘍特異的微小環境適応シグナル伝達を利用した抗がん剤の開発

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研究要旨：低酸素下で盛んに増殖する膵がん細胞の栄養学的基盤としてのオートファジーの関わりを明らかにする目的で Kigamicin D による PANC-1 細胞阻害効果を解析し、グルコース欠乏に依存した酸化ストレス条件下でのユビキチンターンオーバー阻害であることを明らかにした。

A. 研究目的

膵がんに代表されるような低栄養・低酸素条件下で盛んに増殖するがんは、細胞自身のタンパクをターンオーバーする機構であるオートファジーを利用してその産物であるアミノ酸や脂肪酸を代謝してエネルギーを獲得している可能性が指摘されてきた。培養膵がん細胞 PANC-1 を材料として調べたこれまでの研究によって、Kigamicin D はアミノ酸欠乏ではなくグルコース欠乏にのみ応答してそのタンパク分解を有意に阻害することが解った。オートファジーはアミノ酸欠乏下で亢進する特徴があり、Kigamicin D によるタンパク分解阻害はオートファジーへの効果ではなくむしろユビキチン・プロテアソーム系（UPS）への影響と考えられた。実際、グルコース欠乏時に Kigamicin D は PANC-1 細胞質にポリユビキチンを貯留させる効果が有り、阻害率は Lactacystin によるプロテアソーム阻害とほぼ同等という結果を得た。しかしながら、培養条件下で Kigamicin D 処理を行っても、また、in vitro で細胞抽出液に加えた場合でも、プロテアソームのキモトリプシン様あるいはトリプシン様活性にはまったく影響が無かった。また、昨年度研究で脱ユビキチン化酵素への阻害も調べたが、影響はほとんど見られなかった。

グルコース欠乏は細胞のタンパク合成系への負荷を与え、ER ストレスを引き起こすことが知られている。そこでグルコース欠乏下において Kigamicin D がポリユビキチンの蓄積を誘発する機構と ER ストレスとの関係についてさらに解析を行った。

B. 研究方法

1）DNA microarray によるグルコース欠乏下培養 PANC-1 細胞における遺伝子発現の変化解析

透析によってグルコースを除いたウシ胎児血清（FCS）とグルコース不含 DMEM とで再構成したメディウムと通常の高グルコースを含む DMEM/10% FCS それぞれにおいて PANC-1 細胞を 48 時間培養し、RNA を抽出して microarray による遺伝子発現解析を行った。

2）グルコース欠乏下の ER ストレス関連タンパク解析

1）と関連して、glucose±、さらに Kigamicin D 存在下非存在下で PANC-1 細胞を 4 時間培養し、ER ストレス関連タンパクの変化をウェスタンブロットで解析した。ER ストレス関連タンパク質としては、GRP78（BiP）、

calreticulin、CHOP、リン酸化 eIF-2α を対象とした。

（倫理面への配慮）マウス肝細胞単離に際しては、「倫理的基準に基づいたヒト以外の動物種を用いた医学生物学実験の分類」のカテゴリーBに従った。

C. 研究結果

1）グルコース欠乏下における PANC-1 の遺伝子発現変化

PANC-1 をグルコース欠乏培養条件下で 48 時間培養すると、いくつかの ER ストレス関連遺伝子発現に有意な上昇が認められた。すなわち、Dnaj (HSP40) homolog, subfamily C が 2.7 倍、GRP78 (BiP)が 2.3 倍、X-box binding protein 1 が 2.9 倍である。しかし、caspase 等のアポトーシス関連遺伝子の発現はあまり影響されず、グルコース欠乏自体の影響はそれほど大きいとは言えないようである。

2）ER ストレス関連タンパクと Kigamicin D の効果

1）の結果と関連して、タンパクレベルでの ER ストレスの評価を行った。calreticulin と GRP78 はグルコース欠乏下で顕著な増加が認められ、典型的な ER ストレス応答が起こっていることを示唆した。CHOP の発現上昇は見られず、caspase 3 の活性化や eIF-2α のリン酸化も起こっていなかった。グルコース欠乏にさらに Kigamicin D の添加を加えた場合には、唯一認められた変化は eIF-2α のリン酸化が顕著に増加したことである。

D. 考察

microarray 解析から PANC-1 はグルコース欠乏下で穏和な ER ストレスを起こしている状態であることが窺われた。GRP78 と calreticulin が有意に増加している一方、thapsigargin や tunicamycin 処理時のような CHOP の発現上昇や caspase 3 の活性化は認められず、また、eIF-2α のリン酸化も認められなかった。Kigamicin D はグルコース欠乏下でポリユビキチン化タンパクの蓄積を起こすが、Kigamicin D 添加で初めて eIF-2α のリン酸化が認められたことから、eIF-2α のリン酸化に至るシグナル伝達と Kigamicin D 作用の接点が存在すると考えられる。今後さらに検討していく余地がある。

低栄養・低酸素分圧下で増殖する膵がんでは、オートファジーを生存や増殖に活用していると予想していたが、我々の実験ではグルコース欠乏による ER ストレスとの関連でユビキチン・プロテアソーム系への影響を介したタンパク分解阻害という複雑な効果が明らかになった。これまでのアプローチでは、PANC-1 という確立された培養系を用いた好気的条件下の実験データに基づいて解釈を巡らせている。しかし一つ言えることとして、hypoxia における PANC-1 のオートファジーへ Kigamicin D がどのような影響を及ぼすかを知ることが重要であろう。

Kigamicin D の作用点研究は江角班以来の課題であったが、明快な結論を出せないままできている。対がん総合戦略事業の一環として研究を行う立場から Kigamicin D の研究とは独立のアプローチとして抗がん剤の Adriamycin による腎症の機構を明らかにすべく、糸球体微少環境維持に重要な貢献を行っている足場細胞へのアドリアマイシンの阻害効果を調べ、その結果の一部を論文として投稿した。

E. 結論

1．PANC-1 細胞のタンパク分解阻害からは、Kigamicin D はグルコース

欠乏条件下でのみ高分子ポリユビキチン鎖を蓄積させるユニークな作用が見出されたが、その機構についてはERストレスシグナルとの接点に何らかの関わりを持つことが示唆された。

F. 健康危険情報

特になし。

G. 研究発表

論文発表

なし。

現在投稿中のもの

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Miyuki Takagi-Akiba, Katsuhiko Asanuma, Isei Tanida, Norihiko Tada, Juan Alejandro Oliva Trejo, Etsuko Asanuma, Eiki Kominami, Takashi Ueno, Yasuhiko Tomino
Kidney International submitted.

H. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

別紙４

研究成果の刊行に関する一覧表

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Review Article

Preclinical and clinical studies of anticancer agent-incorporating polymer micelles

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The size of anticancer agent-incorporating micelles can be controlled within the diameter range of 20–100 nm to ensure that they do not penetrate normal vessel walls. With this development, it is expected that the incidence of drug-induced side-effects may be decreased owing to the reduced drug distribution in normal tissue. Micelle systems can also evade non-specific capture by the reticuloendothelial system because the outer shell of a micelle is covered with polyethylene glycol. Consequently, a polymer micelle carrier can be delivered selectively to a tumor by utilizing the enhanced permeability and retention effect. Moreover, a water-insoluble drug can be incorporated into polymer micelles. Presently, several anticancer agent-incorporating micelle carrier systems are under preclinical and clinical evaluation. Furthermore, nucleic acid-incorporating micelle carrier systems are also being developed. (*Cancer Sci* 2009; 100: 572–579)

Nanotechnology is one of the fast-moving technologies and is presently contributing significantly to the progress of medical science. Drugs categorized under the drug delivery system (DDS) are made primarily by utilizing nanotechnology. In the field of oncology, DDS drugs have been produced and evaluated in preclinical or clinical trials, with some already approved for clinical use (Table 1). More specifically, DDS can be used for active or passive targeting of tumor tissues. Passive targeting refers to the development of monoclonal antibodies directed against tumor-related molecules, allowing targeting of a tumor from the specific binding of antibodies with respective antigens. However, the application of DDS using monoclonal antibodies is restricted to tumors expressing high levels of related antigens.

Passive targeting can be achieved by utilizing the enhanced permeability and retention (EPR) effect.[1,2] This effect is based on the pathophysiological characteristics of solid tumor tissues, namely, hypervascularity, incomplete vascular architecture, secretion of vascular permeability factors stimulating extravasation within cancer tissue, and absence of effective lymphatic drainage from tumors that impedes the efficient clearance of macromolecules accumulated in solid tumor tissues (Fig. 1A,B).

Several techniques have been developed to maximally utilize the EPR effect such as modification of drug structures and development of drug carriers. Polymeric micelle-based anticancer drugs were originally developed by Kataoka *et al.* in the late 1980s or early 1990s.[3–5] Polymeric micelles were expected to increase the accumulation of drugs in tumor tissues by utilizing the EPR effect as well as to incorporate various kinds of drugs into their inner core with relatively high stability by chemical conjugation or physical entrapment. Also, the size of micelles can be controlled within the diameter range of 20–100 nm to ensure that they do not penetrate normal vessel walls. With this development, it is expected that the incidence of drug-induced side-effects may be decreased owing to the reduced drug distribution in normal tissues.

Table 1. Examples of drug delivery systems in oncology and their stage of development

Passive targeting

Name	Platform	Compound	Clinical stage
NK105	Micelles	Paclitaxel	P2
NC-6004	Micelles	Cisplatin	P1/2
NK012	Micelles	SN-38	P2
Smancs	Polymer conjugate	Neocarzinostatin	Approved
Doxil	Liposome	Doxorubicin	Approved
Abraxane	Albumin particle	Paclitaxel	Approved
Xyota,~	Polymer conjugate	Paclitaxel	P3
CT-2106	Polymer conjugate	Camptothecin	P2
EndoTAG	Cationic Liposome	Paclitaxel	P2
MAG-CPT	Polymer conjugate	Camptothecin	P1
LE-SN-38	Liposome	SN-38	P2
PK1	Polymer conjugate	Doxorubicin	P2
,hT-101	Polymer conjugate	Camptothecin	P2
SP1049C	Micelles	Doxorubicin	P3
CPX-1	Liposome	CPT-11,floxuridine	P2

Active targeting

Name	Platform	Compound	Clinical Stage
Mylotarg	Anti-CD33-Ab	Calicheamicin	Approved
Zevalin	Anti-CD20-Ab	90Y	Approved
Bexxar	Anti-CD20-Ab	131 I	Approved
PK2	Galactose-Polymer	Doxorubicin	P1
MCC465	Ab-liposome	Doxorubicin	P1
MBP-426	Transferrin-liposome	Oxaliplatin	P1
CALAA-01	Transferrin-polymer	siRNA	P1
T-DM1	Anti-HER2-Ab	DM1	P1

In this paper, we review recent developments in polymeric micelle systems presently being evaluated in clinical trials and introduce new micellar formulations.

Anticancer agent-incorporating micelle carrier systems under clinical evaluation

NK105: paclitaxel (PTX)-incorporating micellar nanoparticle

Background. PTX is one of the most useful anticancer agents against various cancers, including ovarian, breast and lung cancers.[6,7] However, it produces serious adverse effects such as neutropenia and peripheral sensory neuropathy. In addition, anaphylaxis and other severe hypersensitive reactions have been reported in 2–4% of patients receiving the drug even after

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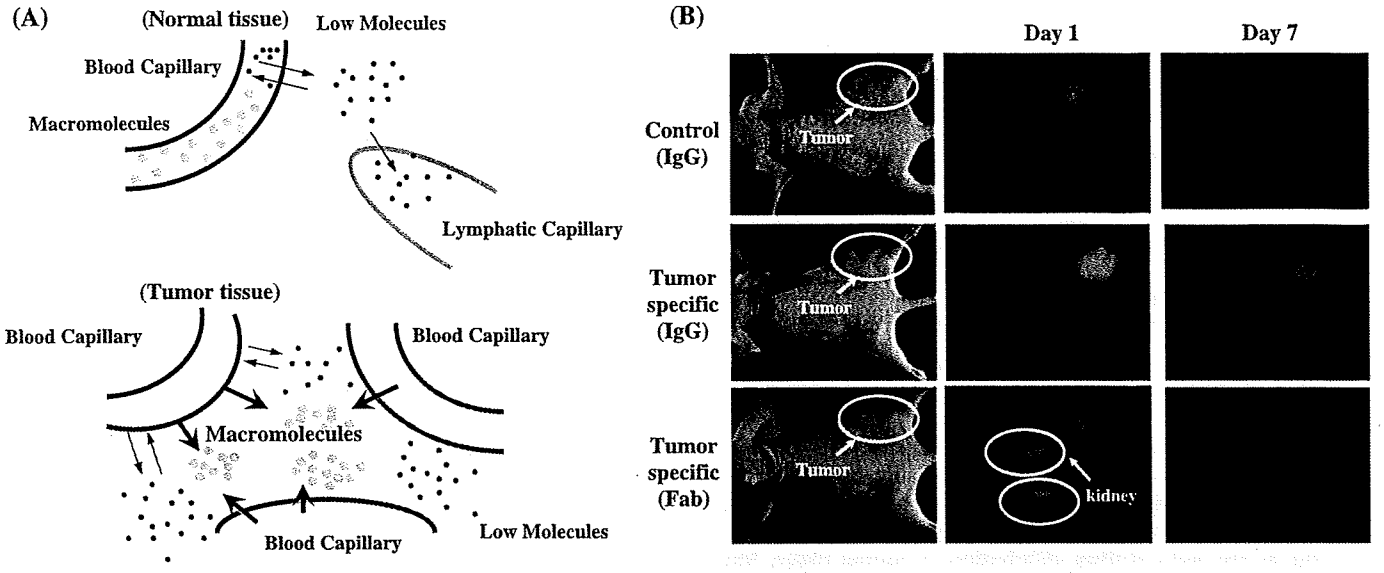

Fig. 1. (A) A diagram of normal and tumor tissue, demonstrating the presence of a lymphatic duct in normal tissue (upper) but the absence of any lymphatic duct in tumor tissue (lower). Small molecules easily leak from normal vessels in the body, which gives small molecules a short plasma half life. On the other hand, macromolecules have a long plasma half life because they are too large to pass through the normal vessel walls, unless they are trapped by the reticuloendothelial system in various organs. In the solid tumor tissues shown in the lower panel, it was found that solid tumors generally possess several pathophysiological characteristics: hypervasculature, secretion of vascular permeability factors stimulating extravasation of macromolecules within the cancer, and absence of effective lymphatic drainage from tumors that impedes the efficient clearance of macromolecules accumulated in solid tumor tissues. These characteristics of solid tumors are the basis of the enhanced permeability and retention (EPR) effect. Summarizing these findings, conventional low-molecular-weight anticancer agents disappear before reaching the tumor tissues and exerting their cell-killing effect. On the other hand, macromolecules and nanoparticles including micelle carriers should have time to reach the tumor, extravasate from the tumor capillaries, and stay for a long time in the tumor tissue, by means of the EPR effect. (B) Example of the EPR effect. This *in vivo* imaging demonstrates that both control whole immunoglobulin G (IgG) and the specific whole monoclonal antibody (mAb) accumulated selectively in the tumor tissue on day 1 after invravenous injection. On day 7, a greater degree of retention of the specific whole mAb as compared to the control IgG was noted. On the other hand, the F(ab) region of the specific mAb with a molecular weight of 45 000 accumulated in the tumor to the same extent as the control whole IgG. Interestingly, fluorescence of the F(ab) could also be detected in both the kidneys, which implied that the antigen binding fragment F(ab) could easily pass through the kidney glomerulus. This accumulation of the control IgG in the tumor represents the EPR effect. The findings suggest that not only the specific affinity of the mAb, but also the size of the molecules and the stability of the molecules in blood are important for tumor-selective targeting.

premedication with anti-allergic agents; these adverse reactions have been attributed to the mixture of Cremophor EL and ethanol used for solubilizing PTX.[8,9] Of the adverse reactions, neutropenia can be prevented or managed effectively by administering a granulocyte colony-stimulating factor. On the other hand, there are no effective therapies to prevent or reduce nerve damage associated with PTX-induced peripheral neuropathy. Thus, neurotoxicity constitutes a significant dose-limiting toxicity of the drug.[10,11]

Preparation and characterization of NK105. To construct NK105 micellar nanoparticles (Fig. 2A), block copolymers consisting of polyethylene glycol (PEG) and polyaspartate,[3–5] were used. PTX was incorporated into polymeric micelles formed by physical entrapment utilizing hydrophobic interactions between PTX and the block copolymer polyaspartate chain. NK105 was prepared by facilitating the self association of NK105 polymers and PTX. NK105 was obtained as a freeze-dried formulation and contained about 23% (w/w) of PTX The weight-average diameter of the nanoparticles was approximately 85 nm with a narrow size distribution.[12]

Preclinical studies. In an *in vivo* pharmacokinetics study using colon 26 tumor-bearing CDF1 mice, the plasma concentration at 5 min (C_{5min}) and the area under the curve (AUC) of NK105 were 11- to 20-fold and 50- to 86-fold higher than those of PTX. The maximum contentration (C_{max}) and AUC of NK105 in colon 26 tumors were approximately 3 and 25 times higher than those of PTX. NK105 continued to accumulate in the tumors until 72 h postinjection (data not shown).

In BALB/c mice bearing subcutaneous HT-29 colon cancer tumors, NK105 exhibited superior antitumor activity compared with PTX ($P < 0.001$). Tumor suppression by NK105 increased in a dose-dependent manner (Fig. 2B).

Paclitaxel treatment has been shown to cause cumulative sensory-dominant peripheral neurotoxicity in humans characterized clinically by numbness and/or paraesthesia of the extremities. Pathologically, axonal swelling, vesicular degeneration, and demyelination have also been observed. We therefore examined the pathologic effects of free PTX and NK105 using both electrophysiological and morphological methods. After drug administration for 2 months, the amplitude of the caudal sensory nerve action potential in the control group increased in association with rat maturation. The amplitude was significantly smaller in the PTX group than in the control group ($P < 0.01$); it was significantly larger in the NK105 group than in the PTX group ($P < 0.05$); and it was comparable between the NK105 group and the control group (Fig. 2C).

Clinical study. A phase I study was designed to determine the maximum tolerated dose (MTD), dose-limiting toxicities (DLTs), and recommended dose (RD) of NK105 for phase II, as well as its pharmacokinetics.[13]

NK105 was administered by intravenous infusion for 1 h every 3 weeks without anti-allergic premedication. The starting dose was 10 mg PTX equivalent/m^2, and the dose escalated according to the accelerated titration method. DLTs were observed in two patients at 180 mg/m^2 (grade 4 neutropenia lasting for more than 5 days), which was determined as MTD. Allergic reactions were not observed in any of the patients except in one. The RD was 150 mg/m^2. A partial response was observed in one pancreatic cancer patient who received more than 12 courses of NK105 (Fig. 2D). Colon and gastric cancer patients experienced

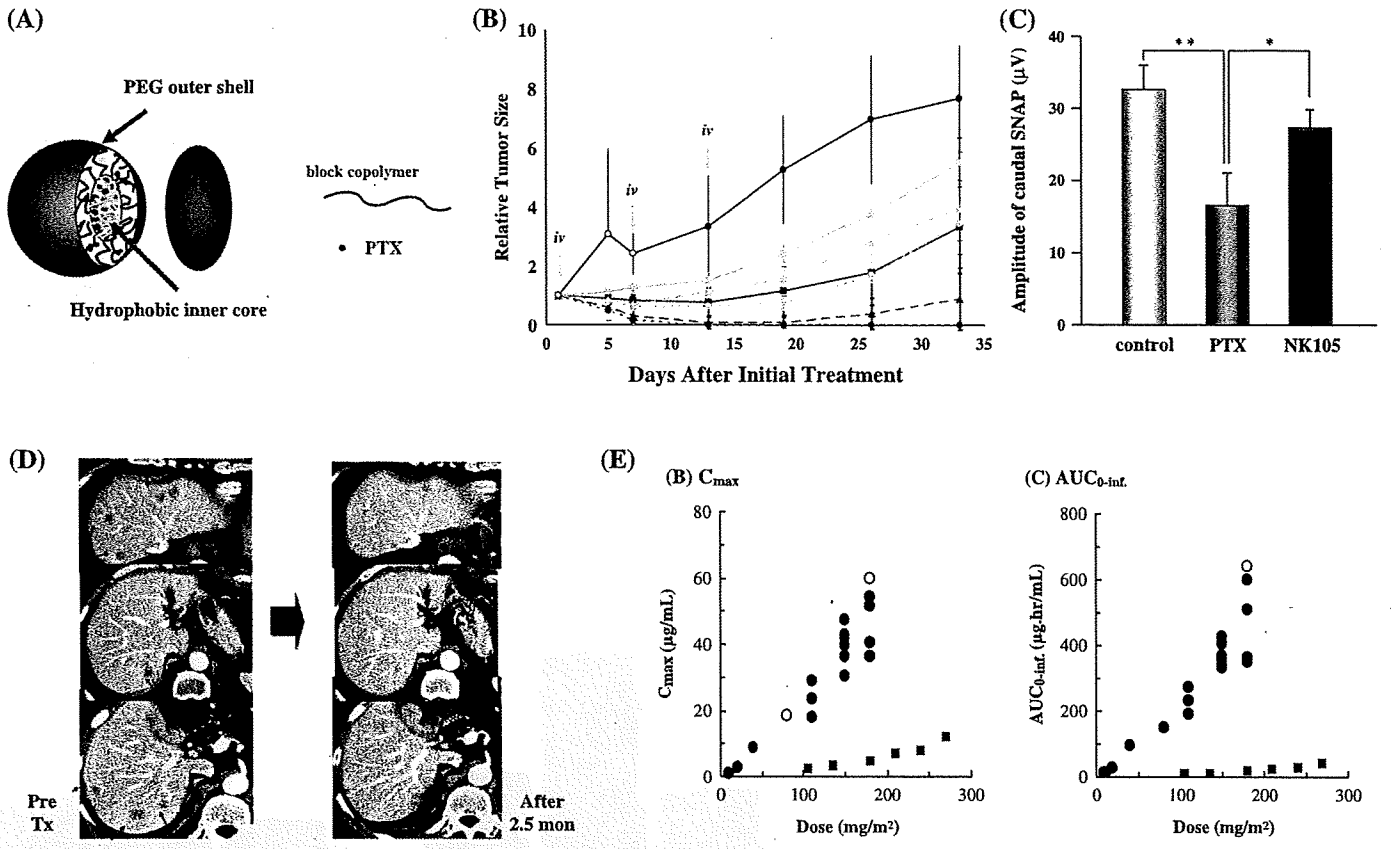

Fig. 2. (A) The schematic structure of NK105, a micelle-forming polymeric drug, that is a polyethylene glycol (PEG)-poly (aspartic acid) block copolymer conjugated with *paclitaxel* (PTX). NK105 was obtained as a freeze-dried formulation and contained approximately 23% (w/w) of PTX. The weight-average diameter of the nanoparticles was approximately 85 nm with a narrow size distribution. (B) Antitumor activity of PTX or NK105 on HT-29 human colon cancer xenografts.Each drug was administered intravenously weekly for 3 times at a PTX equivalent dose of 25 mg/kg, 50 mg/kg, or 100 mg/kg. Light blue lines show the treatment with PTX. Red lines show the treatment with NK105. NK105 exhibited significant superior antitumor activity as compared with PTX at respective dose levels ($P < 0.001$). Especially, tumors disappeared after the first dosing to mice treated with NK105 at 100 mg/kg. Dark blue line (control). Each point: mean ± SE. (C) The amplitude of caudal sensory nerve action potential after the drugs administration for 2 months. The amplitude was significantly smaller in the PTX group as compared to control or NK105 administration group. (D) Serial computed tomography scans. A 60-year-old man with pancreatic cancer who was treated with NK105 at a dose level of 150 mg/m². Baseline scan (left) showing multiple metastasis in the liver. Partial response, characterized by a more than 90% decrease in the size of the liver metastasis (right) compared with the baseline scan. The antitumor response was maintained for nearly 1 year. (E) Correlations between dosage and maximum contentration (C_{max}) and area under the curve (AUC) for both PTX (■) d NK105 (●). Both C_{max} and AUC of NK105 were significantly higher than those of PTX. Different from PTX, the Cmax and AUC of NK105 increased linearly at doses between 10 and 15 mg/m². The AUC of NK105 at the recommended dose was 10–30-fold higher than that of PTX at a conventional dose, 210 mg/m².

stable disease lasting for ten and seven courses, respectively. Despite long-term administration, only grade 1 or 2 neuropathy was observed when the dose or period of drug administration was modified. The C_{max} and AUC of NK105 showed dose-dependent characteristics. The plasma AUC of NK105 at 150 mg/m² was approximately 30-fold higher than that of the commonly used PTX formulation (Fig. 2E).

Dose-limiting toxicity was Grade 4 neutropenia. NK105 facilitates prolonged systemic PTX exposure in plasma. Tri-weekly 1-h infusion of NK105 was feasible and well tolerated, with antitumor activity. A phase II study of NK105 against advanced stomach cancer as a second-line therapy is currently underway.

NC-6004: cisplatin-incorporating micellar nanoparticle

Background. Cisplatin (*cis*-dichlorodiammineplatinum [II]: CDDP] is a key drug in the chemotherapy of various cancers, including lung, gastrointestinal and genitourinary cancers.[14,15] However, it is often necessary to discontinue CDDP treatment because of its adverse reactions (e.g. nephrotoxicity and neurotoxicity) despite its persisting effects.[16] To date, platinum analogs (e.g. carboplatin and oxaliplatin)[17] have been developed to overcome these CDDP-related disadvantages. Consequently, these analogs have become the standard drugs for ovarian[18] and colon[19] cancers. On the other hand, these regimens including CDDP constitute the standard treatment for lung, gastric, testicular[20] and urothelial[21] cancers. Therefore, the development of DDS technology is anticipated, which would enable better selective CDDP accumulation in solid tumors while lessening its distribution in normal tissue.

Preparation and characterization of NC-6004. NC-6004 was prepared according to a slightly modified procedure reported by Nishiyama *et al.*[22] (Fig. 3A). NC-6004 consists of PEG, a hydrophilic chain constituting the outer shell of micelles, and the coordinate complex of poly(glutamic acid) and CDDP, a polymer-metal complex-forming chain constituting the inner core of micelles. A narrowly distributed size of polymeric micelles (20 nm) was confirmed by dynamic light-scattering measurement. Also, static light-scattering measurement revealed that the CDDP-loaded micelles showed no dissociation upon dilution and the critical micellar concentration was $<5 \times 10^{-7}$,

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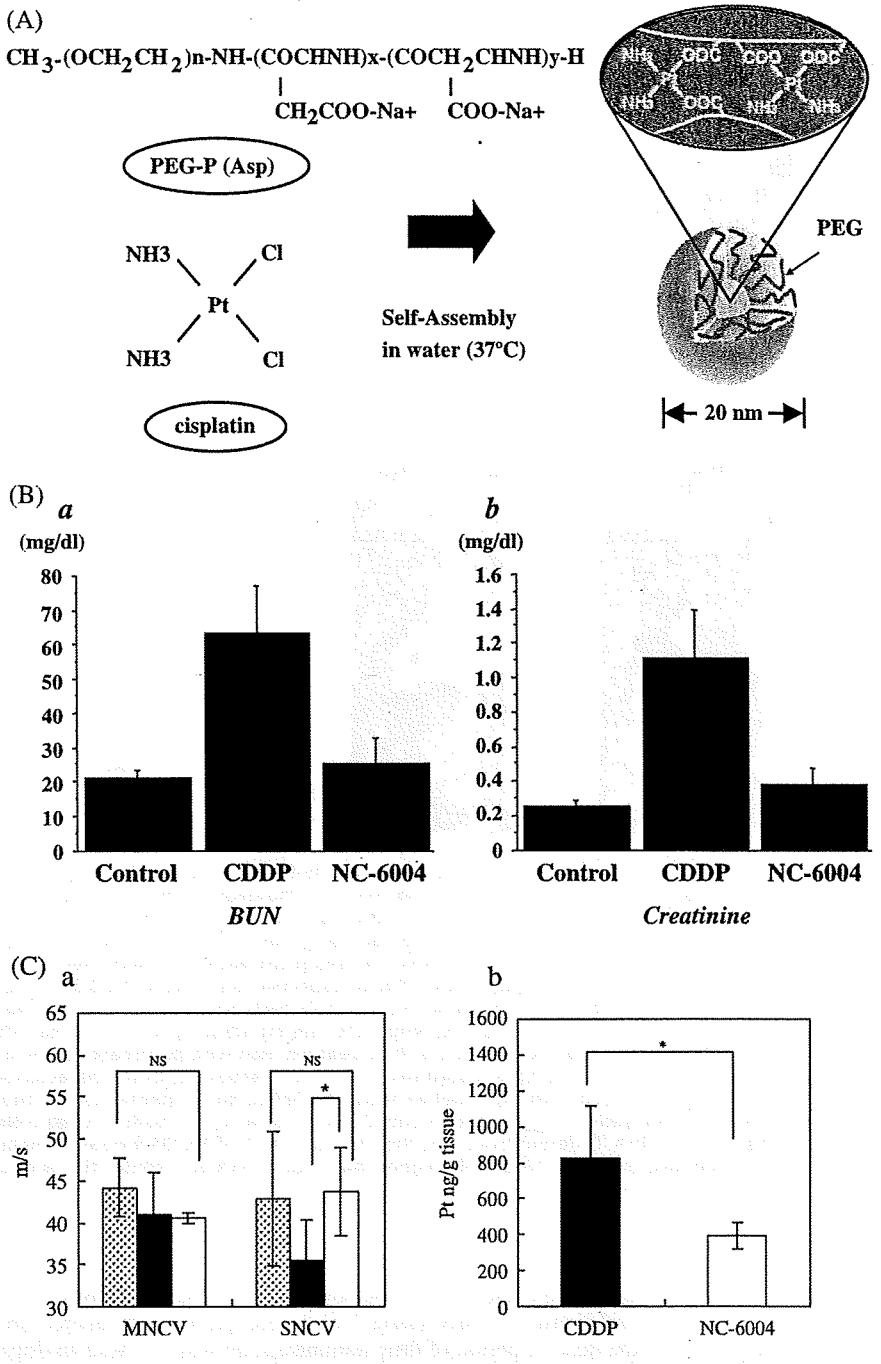

Fig. 3. (A) Cisplatin-incorporating polymeric micelles (NC-6004) consists of polyethylene glycol (PEG), a hydrophilic chain which constitutes the outer shell of the micelles, and the coordinate complex of Poly(Glu) and *cis*-dichlorodiammineplatinum (II) (CDDP) constitutes the inner core of the micelles. The mean particle size of NC-6004 was approximately 20 nm. (B) Nephrotoxicity of CDDP and NC-6004 in rats. The CDDP 10 mg/kg administration group showed significantly higher concentrations of blood urea nitrogen (BUN) and creatinine as compared with the control group and with the NC6004 10 mg/kg administration group. (C) Neurotoxicity of CDDP and NC-6004 in rats. Rats were given 2 mg/kg of CDDP (■) or NC-6004 (□) twice a week, 11 administrations in total. Animals given NC-6004 showed no delay in sensory nerve conductive velocity (SNCV) as compared with the control (¦). On the other hand, animals given CDDP showed a significant delay in SNCV as compared with animals given NC-6004 (A). The analysis by inductively-coupled plasma–mass spectrometer revealed that sciatic nerve concentrations of platinum (Pt) in mice given CDDP were more than 2-fold higher than NC-6004 (b). $*P < 0.05$ NS: not significant.

suggesting remarkable stability compared with typical micelles from amphiphilic block copolymers.[22]

Preclinical study. NC-6004 showed a very long blood retention profile compared with CDDP. The AUC_{0-t} and C_{max} values were significantly higher in animals given NC-6004 than in animals given CDDP, namely, 65-fold and 8-fold, respectively ($P < 0.001$ and $P < 0.001$, respectively). Regarding platinum accumulation in the tumor, platinum concentrations peaked at 10 min following CDDP administration and at 48 h following NC-6004 administration. The C_{max} in the tumor was 2.5-fold higher for NC-6004 than for CDDP ($P < 0.001$). Furthermore, the tumor AUC was 3.6-fold higher for NC-6004 than for CDDP (81.2 μg/mL/h and 22.6 μg/mL/h, respectively).[23]

In nude mice implanted with the human gastric cancer cell line MKN-45, NC-6004 administration groups (5 mg/kg of CDDP) showed no significant difference in tumor growth rate compared with CDDP administration groups. Regarding time-course changes in body weight change rate, the CDDP (5 mg/kg) administration group showed a significant decrease ($P < 0.001$) in body weight compared with the control group. On the other hand, the NC-6004 administration group showed no decrease in body weight compared with the control group (data not shown).

Regarding renal function, the CDDP (10 mg/kg) administration group showed significantly higher plasma concentrations of BUN and creatinine than the control group ($P < 0.05$ and $P < 0.001$, respectively) and NC-6004 (10 mg/kg) administration group ($P < 0.05$ and $P < 0.001$, respectively) (Fig. 3B).

In neurological examination, rats given NC-6004 showed no delay in sensory nerve conductive velocity (SNCV) compared with animals given 5% glucose. On the other hand, rats given CDDP showed a significant delay ($P < 0.05$) in SNCV compared with animals given NC-6004. Sciatic nerve concentrations of

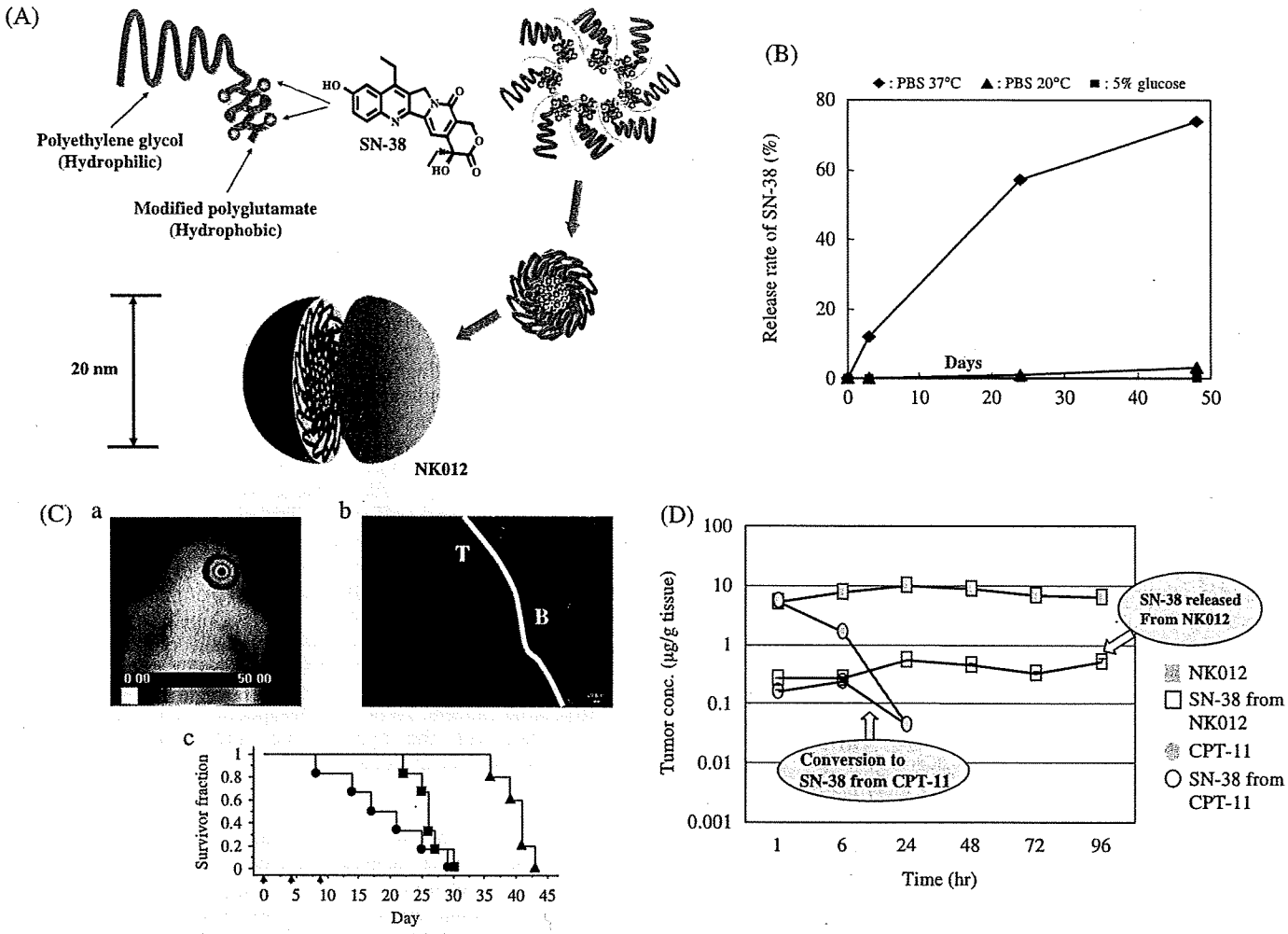

Fig. 4. (A) Schematic structure of NK012. A polymeric micelle carrier of NK012 consists of a block copolymer of polyethylene glycol (PEG) (molecular weight of ≈□5000) and partially modified polyglutamate (≈□ 20 units). Polyethylene glycol (hydrophilic) is believed to be the outer shell and SN-38 was incorporated into the inner core of the micelle. (B) The releasing rates of 7-ethyl-10-hydroxy-CPT (SN-38) from NK012. The data suggested that NK012 is stable in 5% glucose solution before administration and starts to release SN-38, gradually, under physiological conditions after administration. (C) (a) An orthotopic glioma model. Twenty days after U87MG/Luc inoculation. (b) The orthotopic tumor was visualized using a photon imager. The maximum tolerated dose (MTD) of NK012 (30 mg/kg) or Irinotecan hydrochloride (CPT-11) (66.7 mg/kg) was injected intravenously into the tail vein of mice. 24 h after NK012 injection, mice were also administered with fluorescein *Lycopersicon esculentum* lectin (100 μL/mouse) to visualize tumor blood vessels (green). NK012 (blue) was accumulated selectively in tumor tissue. T: tumor, B: normal brain (c) NK012 (30 mg/kg/day), CPT-11 (66.7 mg/kg/day) (■) and saline (●) were intravenously given on days 0 (20 days after tumor inoculation), 4, and 8 (▼). Kaplan-Meier analysis was performed to determine the effect of drugs on time to morbidity, and statistical differences were ranked according to the Mantel-Cox log-rank test using StatView 5.0. (D) Intra-tumor distribution of CPT-11, NK012 (or polymer bound SN-38), and free SN-38 after administration of NK012 and CPT-11 to mice bearing Capan1 xenografts. The time profiles of polymer-bound SN-38 (■), free SN-38 released from NK012 (□), CPT-11 (●), and free SN-38 converted from CPT-11 (○) were obtained by high performance liquid chromatography (HPLC) analysis. The time-points examined were 1, 6, 24, 48, 72, and 96 h after the administration of CPT-11 or NK012.

platinum were significantly ($P < 0.05$) lower in rats given NC-6004 (Fig. 3C). This finding is believed to be a factor that reduced neurotoxicity following NC-6004 administration compared with CDDP administration.

Clinical study. A phase I clinical trial of NC-6004 has recently been completed in the UK.[24] The starting dose of NC-6004 was 10 mg/m^2. NC-6004 was administered once every 3 weeks with only 1000 mL water loading at the day of administration. Administration of doses up to 120 mg/m^2 was performed without inducing significant nephrotoxicity. Although nausea and vomiting are typical CDDP adverse effects, those caused by NC-6004 were generally mild. However, hypersensitivity reactions caused by NC-6004 occurred more frequently than those caused by CDDP. A phase II study will soon be started involving sufficient anti-allergic premedication.

NK012: SN-38-incorporating micellar nanoparticle

Background. Irinotecan hydrochloride (CPT-11) has recently been demonstrated to be active against colorectal, lung, and ovarian cancers.[25–29] CPT-11 is a prodrug and is converted to 7-ethyl-10-hydroxy-CPT (SN-38), a biologically active metabolite of CPT-11, by carboxylesterases (CEs). SN-38 (Fig. 4A) is an analog of the plant alkaloid camptothecin which targets DNA topoisomerase I. SN-38 exhibits up to 1000-fold more potent cytotoxic activity against various cancer cells *in vitro* than CPT-11.[30] Although CPT-11 is converted to SN-38 in the liver and tumors, the metabolic conversion rate is less than 10% of the original volume of CPT-11.[31,32] Moreover, the conversion of CPT-11 to SN-38 depends on the genetic interindividual variability of CE activity.[33] Thus, further efficient use of SN-38 might be of great advantage and may be attractive for cancer

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treatment. The progress of the manufacturing technology of 'micellar nanoparticles' may make it possible to use SN-38 for *in vivo* experiments and further clinical use.

Preparation and characterization of NK012. NK012 is an SN-38-loaded polymeric micelle constructed in an aqueous milieu by the self-assembly of an amphiphilic block copolymer, PEG-PGlu(SN-38).[34] NK012 was obtained as a freeze-dried formulation and contained about 20% (w/w) of SN-38 (Fig. 4A). The mean particle size of NK012 is 20 nm in diameter with a relatively narrow range (Fig. 4A). The releasing rates of SN-38 from NK012 in phosphate buffered saline at 37°C were 57% and 74% at 24 h and 48 h, and those in 5% glucose solution at the same temperature and times were 1% and 3%, respectively (Fig. 4B). These results indicate that NK012 can release SN-38 under neutral conditions even without a hydrolytic enzyme, and is stable in 5% glucose solution. Thus, NK012 is suggested to be stable before administration and starts to release SN-38 gradually under physiological conditions following administration.

Preclinical study. Following CPT-11 injection, the plasma concentrations of CPT-11 and SN-38 rapidly decreased with time in a log-linear fashion. On the other hand, NK012 (polymer-bound SN-38) exhibited slower clearance. In tumor xenografts, NK012 clearance was significantly slower and free SN-38 concentration was maintained for a long time following injection.[34,35] Interestingly, there was no significant difference in the kinetic characteristics of free SN-38 in the small intestine between mice treated with NK012 and CPT-11.

Deviating from the ordinary experimental tumor model, tumors were allowed to grow until they became very large (approximately 1.5 cm), and then treatment was initiated. NK012 showed potent antitumor activity against bulky small cell lung cancer (SCLC) line, SBC-3/Neo, tumors compared with CPT-11. A striking antitumor activity was observed in mice treated with NK012 when we compared its antitumor activity with CPT-11 using SBC-3/VEGF (vascular endothelial growth factor) cells. In the clinical setting, CPT-11/5-FU (fluorouracil) combination therapy is now a standard regimen for colorectal cancer.[25,26] Therefore, it was speculated that the use of NK012 in place of CPT-11 in combination with 5-FU may yield superior results. As expected, the therapeutic effect of NK012/5-FU was significantly superior to that of CPT-11/5-FU against HT-29 xenografts ($P = 0.0004$)[36] (data not shown). In other tumors such as renal cancer,[37] glioma,[38] gastric cancer[39] and pancreatic cancer,[35] NK012 exerted significant superior antitumor activity and induced longer survival compared with CPT-11. In a glioma orthotopic xenograft model, both NK012 and CPT-11 appeared to be able to effectively extravasate from the blood–brain tumor barrier but not from normal brain vessels (Fig. 4C).[38] In a pancreatic cancer cell line, Capan-1 tumor xenograft as a hypovascular tumor model, it was also demonstrated that NK012 showed significantly more potent antitumor activity than CPT-11. Pharmacological examination revealed that only a slight conversion of SN-38 from CPT-11 was observed from 1 h to 24 h, and no SN-38 was detected thereafter. On the other hand, SN-38 released from NK012 continued to be detected from 1 h to 96 h following NK012 injection[35] (Fig. 4D).

Thus, NK012, which combines enhanced distribution with prolonged sustained release of SN-38 within tumors, is ideal for the treatment of hypovascular tumors since the antitumor activity of SN-38 is time-dependent.

Clinical study. Two independent phase I clinical trials have been conducted in the National Cancer Center in Japan[40] and the Sarah Canon Cancer Center in the US[41] in patients with advanced solid tumors to define the MTD, DLT, and recommended phase II dose. NK012 is infused intravenously over 60 min every 21 days until disease progression or unacceptable toxicity occurs. The MTD and RD were 37 mg/m^2 and 28 mg/m^2, respectively. DLT was neutropenia, and diarrhea was mild. The pharmacokinetics (PK) profile in the US study was similar to that in the Japanese study. Antitumor activity was also promising. Partial responses (PR)s were obtained in three patients with triple negative breast cancer, one patient with esophageal cancer and one patient with SCLC. A phase II study in patients with triple negative breast and colorectal cancers will soon be started in the US and Japan.

Future micelle formulations

The development of smart polymeric micelles that dynamically change their properties owing to their sensitivity to chemical or physical stimuli is the most promising trend, leading to the development of targeting therapy with high efficacy and ensured safety.[42] In this way, such micelles can respond to pathological or physiological endogenous stimuli already present in the body or to externally applied stimuli such as temperature, light or ultrasound. One sophisticated and rational formulation approach is to increase the hydrophobicity of the core of the polymeric micelles loaded with a platinum-based drug. This is to modulate the drug-releasing profile induced by chloride ion in the body fluid so as to have a sufficient induction period, and thus avoiding systemic drug leakage and achieving selective and sustained drug release at the site of a solid tumor.[43] Based on this strategy, dichloro (*trans*-l-1,2-diaminocyclohexane) platinum (II) (DachPt)-loaded micelles have recently been developed showing longer life in blood circulation and improved anticancer effects compared with cisplatin-loaded polymeric micelles (Fig. 5A).[44] A pH-triggered system also shows great promise in the treatment of intractable cancers. Here, the therapeutic agent should be stably associated with the hydrophobic core, and drug release is expected to occur with the destabilization of the micelle structure in response to an acidic pH of tumor tissue as well as with disruption of intracellular compartments such as the endosome and lysosome.[45] Notable antitumor efficacy against hypovascular cancer, including pancreatic and diffuse-type gastric cancers of doxorubicin-incorporating polymeric micelles with a pH-responding property was demonstrated to emphasize the promising application of DDS for the treatment of intractable cancers.[46] Photodynamic therapy, which involves the systemic administration of photosensitizers (PSs) and the subsequent photoirradiation of the diseased sites, is a promising physical approach for cancer treatment. However, PSs readily form aggregates, resulting in a significant reduction in singlet oxygen production. To prevent the self-quenching of PSs, ionic dendritic PSs (DPs) have been prepared and incorporated into polymeric micelles (Fig. 5B).[47] Eventually, the DP-loaded micelles showed a significant increase in photocytotoxicity compared with free DPs, achieving a remarkable photodynamic efficacy against a subcutaneous tumor model in experimental animals by systemic injection.

Success in gene and nucleic acid delivery indeed relies on the development of safe and effective carriers. In this regard, polyion complex (PIC) micelles, which are formed between nucleic acid and PEG-polycation block copolymers, have received much attention owing to their small size (~100 nm) and excellent biocompatibility.[48] Recently, an siRNA-incorporating PIC micelle has been prepared from the PEG-poly(lysine) block copolymer with sulthydryl (SH) groups in the side chain (Fig. 5C).[49] This PIC micelle is expected to release the loaded siRNA selectively in the cytoplasm by cleavage of disulfide cross-linking in response to a reductive intracellular environment, leading to the effective silencing of target genes related to oncogenesis. On the other hand, the disulfide cross-linking is sufficiently stable in the blood compartment, enabling prolonged circulation because of the oxidized atmosphere in the body. Furthermore, facilitated endosomal escape occurs in the PIC micelle having an intermediated layer between the shell and the core phase to exert selective destablization of the endosomal membrane through the proton

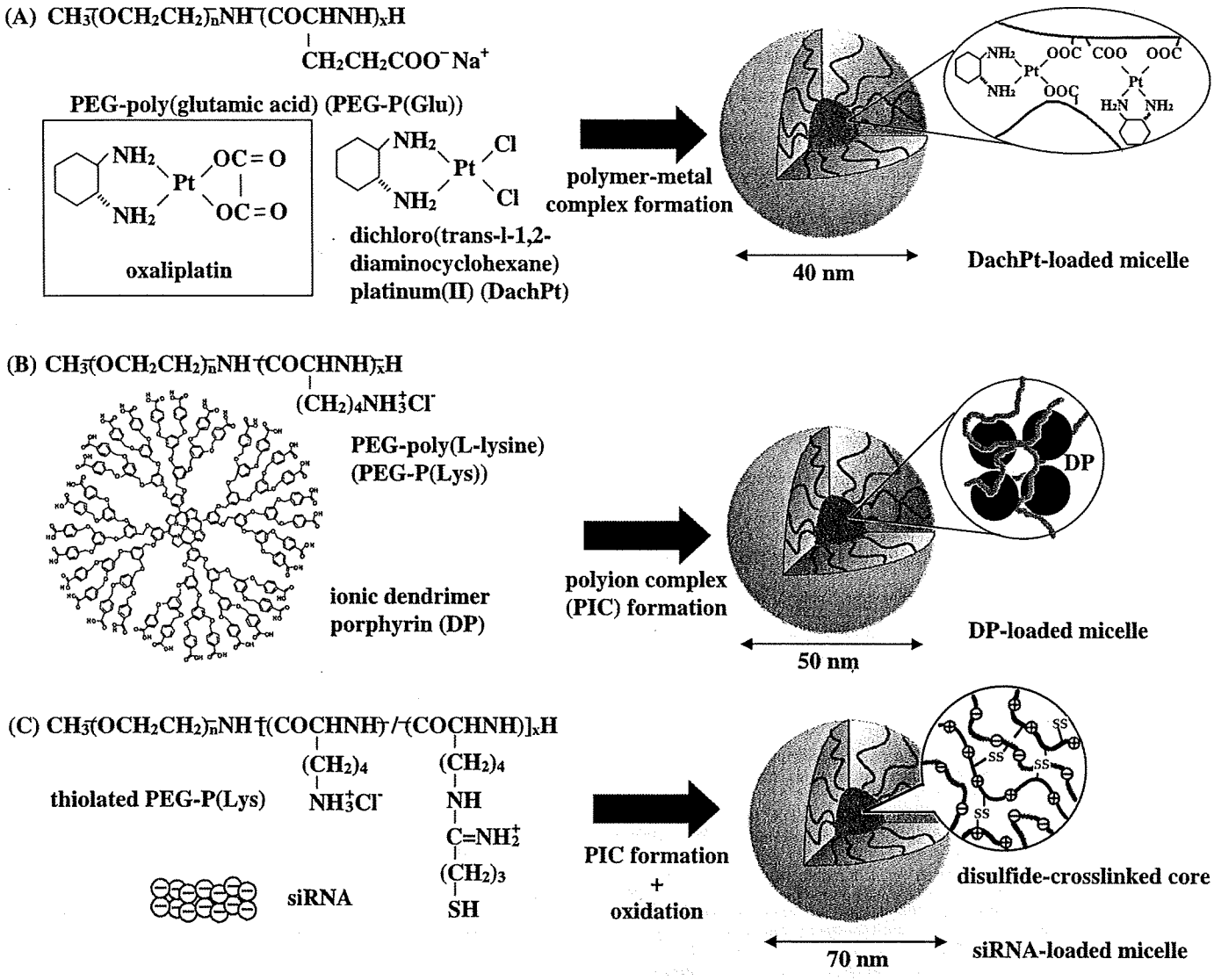

Fig. 5. Formation of polymeric micelles incorporating dichloro (*trans*-l-1,2-diaminocyclohexane) platinum (II) (DachPt) (A), ionic dendritic photosensitizers (B) and siRNA (C).

sponge effect as well as direct perturbation of the membrane structure.[50] The evaluation of these PIC micelles loaded with plasmid DNA or siRNA will soon be performed *in vivo* for future molecular therapy.

Conclusion

Presently, the phrase EPR proposed by Maeda *et al.* has become a fundamental principle in the field of DDS. Until recently, the EPR effect has not been recognized in the field of oncology; however, some oncologists have now become more acquainted with this effect since some drugs such as doxil, abraxane and several PEGylated proteinacious agents formulated based on the EPR effect have been approved in the field of oncology. Micelle carrier systems described here are obviously categorized as DDS based on the EPR effect. We believe that some anticancer agent-incorporating micelle nanoparticles may soon be approved for clinical use.

Acknowledgments

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