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別紙１

厚生労働科学研究費補助金

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腫瘍細胞選択的新規抗がん剤としての葉酸修飾メチル-β-シクロデキストリンの細胞死誘導機構の解明

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研究代表者　本山　敬一

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別紙３

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）
総括研究報告書

研究課題：腫瘍細胞選択的新規抗がん剤としての葉酸修飾メチル-β-シクロデキストリンの細胞死誘導機構の解明

研究代表者：本山敬一　熊本大学大学院生命科学研究部　製剤設計学分野

研究要旨

本研究の目的は、腫瘍細胞選択的新規抗がん剤として期待される葉酸修飾メチル-β-シクロデキストリン（FA-M-β-CyD）の抗腫瘍活性および細胞死誘導機構を明らかにすることである。種々検討した結果、FA-M-β-CyD は葉酸レセプター（FR-α）を介して細胞会合し、抗腫瘍活性を示すことが明らかとなった。さらに、誘導される細胞死について検討を行ったところ、FA-M-β-CyD は、アポトーシスではなく、オートファジーを誘導することが示唆された。また、FA-M-β-CyD はミトコンドリア膜電位を著しく上昇させたことから、ミトコンドリアにストレスを与え、マイトファジーを誘導した可能性が考えられる。次に、FA-M-β-CyD の *in vivo* 抗腫瘍活性を検討するため、担がんマウスに FA-M-β-CyD を尾静脈内に単回投与し、腫瘍体積および生存率について検討した。コントロール群と比較して、FA-M-β-CyD 投与群では顕著に腫瘍の成長を抑制した。さらに、コントロール群では、担がんマウスは癌細胞を移植後 70 日目までに全例死亡したのに対して、FA-M-β-CyD 投与群では、140 日目においても 100% の生存率を示した。また、ヒトメラノーマを異種移植した担がんマウスに対しても、抗腫瘍効果を示す傾向が確認された。これらの結果より、FA-M-β-CyD は *in vivo* においても優れた抗腫瘍活性を有することが示唆された。以上の知見より、FA-M-β-CyD は、細胞形質膜上の FR-α を介して細胞内に取り込まれた後、ミトコンドリアの膜電位を上昇させることでストレスを誘導し、オートファゴソームの形成およびマイトファジーを介して抗腫瘍活性を示したものと考えられる。

A. 研究目的

本研究の目的は、腫瘍細胞選択的新規抗がん剤として期待される葉酸修飾メチル-β-シクロデキストリン（FA-M-β-CyD）の抗腫瘍活性および細胞死誘導機構を明らかにすることである。

メチル-β-シクロデキストリン（M-β-CyD）は、腫瘍細胞で発現が上昇する脂質ラフトからコレステロールを遊離させることにより、その構造や機能に影響を与えるラフト阻害剤である。我々は、M-β-CyDは形質膜上の脂質ラフトからコレステロールを遊離させることにより、PI3K-Akt-Bad 経路を介した細胞生存シグナルを阻害し、アポトーシスを誘導することを明らかにした（*Eur. J. Pharm. Sci.*, 2009）。一方、葉酸レセプター（FR-α）は各種上皮がんで過剰発現しているため、葉酸（FA）はがん標的リガンドとして汎用されている。これまで我々は、M-β-CyDのがん細胞選択性および抗腫瘍効果の増大を企図して、M-β-CyD に FA を導入したFA-M-β-CyDを新規に調製し、FA-M-β-CyDが FR-α 高発現細胞に対して殺細胞効果を示すことを明らかにした。そこで本研究では、FA-M-β-CyD の抗腫瘍活性および細胞死誘導機構について検討した。

B. 研究方法

FA-M-β-CyD の細胞会合は、TRITC を付加したTRITC-FA-M-β-CyD を用いて、フローサイトメトリーにて検討した。FA-M-β-CyD により誘導される細胞死がアポトーシスであるか否かを DNA 含量およびミトコンドリア膜電位を指標に検討した。オートファゴソーム形成に及ぼす FA-M-β-CyD の影響は、オートファゴソームマーカー分子である LC3-II を Cyto-ID® を用いて染色し、蛍光顕微鏡により観察した。FA-M-β-CyD の*in vivo* 抗腫瘍活性は、Colon-26 細胞（FR-α(+)）および Ihara細胞（FR-α(+)）を用いて作成した担がんマウスに FA-M-β-CyDを尾静脈内に単回投与し、腫瘍体積および生存率について検討した。

なお、本研究では、組換えDNA実験、遺伝子治療臨床研究、特定胚を取り扱う研究、ヒトES細胞の研究、ヒトゲノム・遺伝子解析研究、疫学

研究、臨床研究に該当する研究を計画しておらず、倫理面・安全面において問題はない。また、動物実験は、熊本大学動物実験等に関する規則に則り、動物実験計画書の作成・提出、実験動物実験安全管理委員会での審議を経て、学長からの許可のもと実験を行った。なお、本研究を推進する上で、動物を用いた前臨床試験の実施例は必要であるが、必要最低限の動物を用いて、動物に可能な限り苦痛を与えないように研究を実施した。

C. 研究結果

C-1. FA-M-β-CyD の *in vitro* 抗腫瘍活性誘導機構

C-1-1. FA-M-β-CyD の細胞会合および細胞内取り込み

一般に、CyD は親水性かつ分子量が約 1000 と大きいことから細胞内に取り込まれにくいことが知られている。実際、M-β-CyD は細胞内にほとんど取り込まれないことを我々は明らかにしている。しかし、FA-M-β-CyD は FR-α 高発現細胞選択的に優れた抗腫瘍活性を有すること、さらに、その抗腫瘍活性誘導に FR-α を介した細胞内取り込みが関与することが示唆された。そこで本項では、TRITC を付加した TRITC-FA-M-β-CyD を用いて、各種 FR-α 高発現細胞との細胞会合に及ぼす FR 競合阻害剤の影響を検討した。また、FR-α ノックダウン KB 細胞を用いて、TRITC-FA-M-β-CyD の細胞会合に及ぼす FR-α の影響も検討した。実験は 4 mM FA 存在下、10 μM TRITC-FA-M-β-CyD 添加後 1 時間における TRITC の蛍光強度をフローサイトメトリーおよび蛍光顕微鏡により評価した。また、細胞内分布を蛍光顕微鏡にて観察した。さらに、KB 細胞 (FR-α (+)) および A549 細胞 (FR-α (-)) を 1 mM FA-M-β-CyD 含有無血清培地で 1 時間処理した後、1 M 水酸化ナトリウムで細胞を可溶化し、細胞内に取り込まれた FA の蛍光強度を蛍光分光光度計にて評価した。

TRITC-FA-M-β-CyD は KB 細胞、Ihara 細胞および M213 細胞と会合することが示唆された。また、TRITC-FA-M-β-CyD のヒストグラムのピークは、FA 添加により左側にシフトした。また、FR-α ノックダウン細胞においても TRITC-FA-M-β-CyD の細胞会合は抑制されることが示唆された。また、蛍光顕微鏡による観察から、TRITC-FA-M-β-CyD は KB 細胞内に取り込まれることが確認された。

さらに、FA の蛍光強度を指標に、FA-M-β-CyD の細胞会合量を調べたところ、KB 細胞に対する FA-M-β-CyD の会合量は、A549 細胞の系よりも有意に高いことが示唆された。

これらの結果より、FA-M-β-CyD は FR-α 高発現細胞と FR-α を介して細胞会合することが強く示唆された。さらに、TRITC-FA-M-β-CyD は添加 1 時間後において細胞質に TRITC 由来の蛍光が観察されたことから、M-β-CyD とは対照的に細胞内に取り込まれることが確認された。

C-1-2. DNA 含量およびミトコンドリア膜電位変化に及ぼす FA-M-β-CyD の影響

これまで我々は、KB 細胞において M-β-CyD はアポトーシスを誘導することを明らかにしている。そこで本項では、FA-M-β-CyD により誘導される細胞死がアポトーシスであるか否かを DNA 含量およびミトコンドリア膜電位変化を指標に検討した。KB 細胞に M-β-CyDs (10 mM) を 2 時間適用後の DNA 含量を調べたところ、DM-β-CyD 処理では、DNA 含量の有意な低下が認められた。一方、M-β-CyD および FA-M-β-CyD 処理では、コントロールとほぼ同程度の DNA 含量を示した。

各種 M-β-CyDs (10 mM) を 2 時間適用後のミトコンドリア膜電位および FA 添加の影響を調べたところ、DM-β-CyD および M-β-CyD の添加により、KB 細胞のミトコンドリア膜電位が有意に低下した。一方、FA-M-β-CyD 処理ではミトコンドリア膜電位は顕著に上昇した。また、FA の添加により、FA-M-β-CyD により惹起されたミトコンドリア膜電位の上昇はコントロールレベルまで低下した。

これらの結果より、FA-M-β-CyD は KB 細胞に対して、アポトーシスを誘導しない可能性が示唆された。

C-1-3. カスパーゼ-3/7 の活性化に及ぼす FA-M-β-CyD の影響

前項での検討に引き続き、本項ではアポトーシス実行因子であるカスパーゼ-3/7 活性に及ぼす M-β-CyDs の影響を調べた。なお、本項ではカスパーゼ-3/7 の活性化により生じるアミノルシフェリンの蛍光を指標に、アポトーシス誘導能を蛍光顕微鏡にて観察した。DM-β-CyD 添加によりカスパーゼ-3/7 活性に由来する蛍光強度の増加が観察された。一方、M-β-CyD および

FA-M-β-CyD 添加による蛍光強度の増加は認められなかった。これらの結果より、DM-β-CyD はアポトーシスを惹起するのに対し、FA-M-β-CyD はアポトーシス非依存的経路を介して細胞死を誘導することが示唆された。また、本実験条件下、M-β-CyD は細胞死を誘導しないことが示唆された。

C-1-4. オートファゴソーム形成に及ぼす FA-M-β-CyD の影響

前項での検討より、FA-M-β-CyD はアポトーシス非依存的経路により抗腫瘍活性を誘導することが明らかとなった。そこで本項では、オートファゴソーム形成に及ぼす FA-M-β-CyD の影響を検討した。なお、オートファゴソームは、マーカー分子である LC3-II を Cyto-ID® を用いて染色し、蛍光顕微鏡により観察した。M-β-CyD は適用後 2 時間で LC3-II 由来の蛍光は観察されなかった。一方、FA-M-β-CyD では、LC3-II 由来の蛍光が顕著に増大した。さらに、オートファジー阻害剤である LY294002 の添加により、LC3-II 由来の蛍光は著しく減弱した。これらの結果より、FA-M-β-CyD は、オートファジーを誘導することが示唆された。

C-1-5. FA-M-β-CyD の抗腫瘍活性に及ぼすオートファジー阻害剤添加の影響

前項において、FA-M-β-CyD はオートファゴソームの形成を誘導することが示唆された。そこで本項では、FA-M-β-CyD により誘導される抗腫瘍活性がオートファジーであるか否かを検討した。今回、オートファジー阻害剤には、バフィロマイシン A_1 およびクロロキンを用いた。バフィロマイシン A_1 は、マクロライド系抗生物質の一つであり、V-ATPase を持つリソソームやエンドソームの内部酸性化を阻害する。その結果、オートファゴソームとリソソーム/エンドソームとの融合が阻害され、細胞質にオートファゴソームが蓄積し、オートリソソームが減少する。一方、クロロキンは、V-ATPase 活性非依存的にリソソームの pH を上昇させ、オートファゴソームとの融合を阻害する。

オートファジー阻害剤を前処理後、FA-M-β-CyD (5 mM) を 2 時間処理後の抗腫瘍活性を評価した。その結果、FA-M-β-CyD の抗腫瘍活性は、バフィロマイシン A_1 およびクロロキン添加により低下した。これらの結果より、FA-M-β-CyD の抗腫瘍活性はオートファジーを介することが示唆された。

C-1-6. FA-M-β-CyD 処理後のオートファゴソームとミトコンドリアとの共局在

前項までの検討において、FA-M-β-CyD は、オートファジーを介した抗腫瘍活性を誘導することが示唆された。さらに、FA-M-β-CyD はミトコンドリア膜電位を著しく上昇させたことから、ミトコンドリアにストレスを与え、マイトファジーを誘導した可能性が考えられる。そこで本項では、各種 FR-α 高発現細胞を用いて、FA-M-β-CyD のマイトファジー誘導能について検討した。実験は、LC3-II を蛍光ラベル化し、さらにローダミン 123 にてミトコンドリア膜を染色後、蛍光顕微鏡にてオートファゴソームとミトコンドリアとの共局在を観察した。

KB 細胞を DM-β-CyD および M-β-CyD (5 mM) で 2 時間処理したところ、LC3-II の蛍光強度の増大は観察されなかった。一方、FA-M-β-CyD 処理では、LC3-II の蛍光強度の有意な上昇が観察された。さらに、LC3-II とローダミン 123 の蛍光が一部共局在したことから、オートファゴソーム内にミトコンドリアが取り込まれている可能性が示唆された。同様な結果が、Ihara 細胞および M213 細胞においても確認された。

これらの結果より、FA-M-β-CyD は FR-α 高発現細胞においてマイトファジーを誘導することが示唆された。

C-2. FA-M-β-CyD の *in vivo* 抗腫瘍活性

前節までの検討において、FA-M-β-CyD は FR-α 高発現細胞選択的にオートファジーを介した抗腫瘍活性を誘導することが示唆された。そこで本節では、FA-M-β-CyD の *in vivo* 抗腫瘍活性を検討するため、Colon-26 細胞 (FR-α (+)) および Ihara 細胞 (FR-α (+)) を用いて作成した担がんマウスに FA-M-β-CyD を腫瘍内および尾静脈内に単回投与し、腫瘍体積、体重および生存率について検討した。なお、Ihara 細胞を皮下移植した BALB/c Rag-2/Jak3 ノックアウトマウスは、B 細胞、T 細胞、NK 細胞が完全に欠損した高度免疫不全マウスである。

Colon-26 細胞を皮下に同種移植した担がんマウス腫瘍内投与後の腫瘍体積および体重変化を調べたところ、コントロール群と比較して、FA-M-β-CyD 投与群は顕著に腫瘍の成長を抑制した。また、コントロール群の体重は、腫瘍体積の増大に伴い顕著に増加した。一方、

FA-M-β-CyD 投与群では、腫瘍体積の有意な減少により体重増加は緩やかであった。　同担がんマウス尾静脈内に単回投与後の腫瘍体積変化を調べたところ、コントロール群と比較して、FA-M-β-CyD 投与群では顕著に腫瘍の成長を抑制した。さらに、投与時には約 8 mm あった腫瘍は、FA-M-β-CyD 処理後 7 日目には完全に消失した。また、コントロール群では腫瘍体積の増加に伴い、体重の顕著な増加が認められたが、FA-M-β-CyD 投与群では、体重の増加は緩やかであった。さらに、コントロール群では、Colon-26 細胞を移植後 70 日目までに全例死亡したのに対して、FA-M-β-CyD 投与群では、140 日目においても 100% の生存率を示した。

ヒトメラノーマ Ihara 細胞を皮下に異種移植した担がんマウス尾静脈内に単回投与後の腫瘍体積変化を調べたところ、コントロール群と比較して、FA-M-β-CyD 投与群では腫瘍の成長を抑制する傾向が確認された。また、コントロール群では体重の顕著な低下が認められたが、FA-M-β-CyD 投与群では、体重の減少は緩やかであった。

これらの結果より、FA-M-β-CyD は *in vivo* においても優れた抗腫瘍活性を有することが示唆された。

D. 考察

これまで我々は、M-β-CyD は細胞形質膜上のリピッドラフトからコレステロールを漏出させることにより、DNA 含量およびミトコンドリア膜電位を低下させ、アポトーシスを誘導することを明らかにした。一方、FA-M-β-CyD は FR-α の発現の有無に関わらず、DM-β-CyD および M-β-CyD よりも高いコレステロール漏出作用を示した。しかし、興味深いことに KB 細胞において FA-M-β-CyD は DNA 含量を低下させず、ミトコンドリア膜電位の顕著な上昇を誘導した。さらに、FA-M-β-CyD はカスパーゼ-3/7 の活性化も誘導しなかったことから、FA-M-β-CyD の抗腫瘍活性はアポトーシス非依存的経路を介することが強く示唆された。また、前述したように、FA-M-β-CyD は FR-α 低発現細胞である A549 細胞においても優れたコレステロール漏出効果を示したが、抗腫瘍活性はほとんど認められなった。これらの結果より、FA-M-β-CyD の抗腫瘍活性に対する細胞形質膜との相互作用の寄与は低いものと考えられる。

オートファジーとは、細胞内成分が二重膜によって周囲から隔離され、リソソームと融合することによって消化される細胞内浄化機構である。オートファジーは、定常状態の細胞では軽度に活性化されており、細胞構成成分を少しずつ分解することにより細胞の新陳代謝に貢献している。一方、細胞に何らかの刺激が加わると、これに対応するために大規模なオートファジーが誘導される。本研究において、FA-M-β-CyD は KB 細胞においてオートファゴソームを形成すること、また、オートファジー阻害剤であるクロロキンやバフィロマイシン A_1 の前処理によって、その抗腫瘍活性が著しく減弱したことから、FA-M-β-CyD はオートファジーを誘導することが強く示唆された。FA-M-β-CyD によるオートファジーの誘導は、前述したように、ミトコンドリア膜電位の上昇に示されるストレスに起因するものと推察される。近年、ミトコンドリア膜にもリピッドラフトが存在しており、ミトコンドリアの機能や細胞死を制御していることが報告された。また、Ziolkowski らはラット肝臓から回収したミトコンドリアを M-β-CyD で処理すると、リピッドラフトからコレステロールが漏出され、ミトコンドリアのエネルギー代謝や膨潤などの形態学的変化が抑制されることを明らかにした。さらに、ミトコンドリア外膜のリピッドラフトには、膜電位を調節する VDAC が局在しており、pore の開閉を調節してミトコンドリアと細胞質間の ATP や呼吸基質などの物質輸送を行っている。これらの報告より、細胞膜上の FR-α を介して細胞内に取り込まれた FA-M-β-CyD は、何らかの方法でエンドソームを脱出した後、ミトコンドリアのリピッドラフトからコレステロールを漏出させることにより、そのエネルギー代謝や膜電位調節機能を破綻させ、オートファジーを誘導した可能性が考えられる。一方、M-β-CyD は細胞内にほとんど取り込まれないために、細胞形質膜からのコレステロールの漏出を介してアポトーシス誘導へと向かうが、ミトコンドリア膜とは相互作用できず、オートファジーを誘導しなかったものと推察される。

近年、ミトコンドリアはアポトーシスの制御のみならずオートファジー様細胞死誘導にも関わることが明らかとなっている。ミトコンドリアの品質管理としては、1) プロテアーゼによるミトコンドリア内タンパク質の分解、2) ミトコンドリアそのものの分解、3) ミトコンドリアのダイナミックな分裂と融合による不良なミトコンドリアの隔離、などが知られている。なかでも、ミトコンドリアそのものを分解する経路として、ミトコンドリア選択的オートファジーで

あるマイトファジーが報告されている。今回、FA-M-β-CyD 処理により形成されたオートファゴソームとミトコンドリアは一部共局在したことから、マイトファジーの関与が示唆された。近年、Narendra らは、Parkin および PTEN-induced putative kinase protein 1 (PINK-1) が異常なミトコンドリア外膜に結合し、マイトファジーを誘導することを報告している。また、神吉らはミトコンドリア外膜タンパク質である Atg32 が、マイトファジーの基質として必須であることを明らかにしている。今後、Parkin や PINK-1、Atg32 の発現や機能に及ぼす FA-M-β-CyD の影響を詳細に検討する必要がある。

Colon-26 細胞 (FR-α (+)) を同種移植した BALB/c マウスおよび Ihara 細胞 (FR-α (+)) を異種移植した BALB/c Rag-2/Jak3 KO マウスに FA-M-β-CyD を静脈内に単回投与したところ、腫瘍の成長を有意に抑制し、マウスの生存率を著しく改善することが明らかとなった。本実験で使用した BALB/c Rag-2/Jak3 KO マウスは、岡田らによって開発された、成熟 T 細胞、B リンパ球のみならず NK 細胞も欠失した高度免疫不全マウスであり、異種移植モデルマウスとして有用である。今回、FA-M-β-CyD は同種移植した担がんマウスのみならず、ヒト由来 Ihara 細胞 (FR-α (+)) を異種移植した BALB/c Rag-2/Jak3 KO マウスに対しても、優れた抗腫瘍効果を示したことから、今後、臨床試験への応用が期待される。

E. 結論

FA-M-β-CyD は、*in vitro* および *in vivo* において FR-α 高発現細胞選択的抗がん剤として有用であり、その抗腫瘍効果に FR-α を介したがん細胞選択的な取り込みおよびオートファジーの関与が示唆された。

F. 健康危険情報

本研究を通じて、健康危険に関する情報は特に得られていない。

G. 研究発表

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Involvement of Autophagy in Antitumor Activity of Foalte-appended Methyl-b-cyclodextrin., R. Onodera, K. Motoyama, N. Tanaka, A. Ohyama, A. Okamatsu, T. Higashi, R. Kariya, S. Okada, H. Arima., *Sci. Rep.*, **4**, 4417, 1-8 (2014).

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H. 知的財産権の出願・登録状況

1. 特許取得
   名称：腫瘍細胞選択的抗がん剤
   発明者：有馬英俊、本山敬一、東大志
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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レイアウト

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
R. Onodera, K. Motoyama, A. Okamatsu, T. Higashi, R. Kariya, S. Okada, H. Arima	Involvement of Cholesterol Depletion from Lipid Rafts in Apoptosis Induced by Methyl-β-cyclodextrin	International Journal of Pharmaceutics	452	116-123	2013
R. Onodera, K. Motoyama, N. Tanaka, A. Ohyama, A. Okamatsu, T. Higashi, R. Kariya, S. Okada, H. Arima	Involvement of Autophagy in Antitumor Activity of Foalte-appended Methyl-β-cyclodextrin	Scientific reports	4	1-8	2014

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Involvement of cholesterol depletion from lipid rafts in apoptosis induced by methyl-β-cyclodextrin

Risako Onodera[a], Keiichi Motoyama[a], Ayaka Okamatsu[a], Taishi Higashi[a], Ryusho Kariya[b], Seiji Okada[b], Hidetoshi Arima[a,*]

[a] Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Chuo-ku, Kumamoto 862-0973, Japan
[b] Division of Hematopoiesis, Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Chuo-ku, Kumamoto 860-0811, Japan

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ABSTRACT

Methyl-β-cyclodextrin (M-β-CyD), which is widely used as a lipid rafts disrupting agent, is known to induce cytotoxicity at high concentration. In the present study, we investigated the potential of M-β-CyD as an antitumor drug. M-β-CyD markedly caused apoptotic cell-death in KB cells, a human oral squamous carcinoma cell line, Ihara cells, a highly pigmented human melanoma cell line, and M213 cells, a human cholangiocarcinoma cell line, through cholesterol depletion in cell membranes. The DNA content and mitochondrial transmembrane potential in KB cells were significantly decreased after treatment with M-β-CyD. Additionally, M-β-CyD elevated the caspase-3/7 activity in KB cells. Meanwhile, M-β-CyD did not induce the formation of autophagic vacuoles in KB cells. M-β-CyD drastically inhibited the tumor growth after intratumoral injection to Colon-26 cells-bearing mice. These results strongly suggest that M-β-CyD induced apoptosis in tumor cells and had the potential a novel antitumor agent and/or its lead compound.

1. Introduction

There are four forms of cell death: apoptosis, autophagic cell-death, necrosis and programmed necrosis. Among the four distinct forms of cell death, apoptosis is best studied. Apoptosis is roughly defined by biochemical and morphological changes of the cells (Taatjes et al., 2008). Two distinct apoptotic signaling pathways, *i.e.* mitochondrial-dependent and -independent pathways regulate apoptosis activation. Intracellular cues, such as damage to the cell's DNA, drive apoptosis primarily through the mitochondrial-dependent pathway, while extracellular signals; usually generated by cytotoxic cells of immune system, trigger apoptosis mainly through the mitochondrial-independent pathway (Ashkenazi, 2008). Both pathways stimulate pro-apoptotic caspase and are activated through a process called 'the caspase cascade' (Lavrik et al., 2005). Acceleration of these apoptotic signals in cancer cells is one of strategy for anti-cancer therapy.

Membrane lipids are known to be associated with cell-death. Cholesterol is an abundant component of the plasma membrane of eukaryotic cells, which plays a pivotal role in the regulation of membrane fluidity, permeability, receptor function and ion channel activity (Burger et al., 2000). The lateral distribution of cholesterol in the membrane is not uniform and its content is particularly high in lipid rafts. These microdomains have been reported to act as molecular platforms that spatially organize membrane receptor molecules such as epidermal growth factor (EGF) receptor and CD95 (Fas) (Simons and Toomre, 2000). This reorganization is of crucial importance in the initiation and regulation of inflammatory processes and apoptosis. For example, redistribution of death receptors such as Fas to cholesterol-enriched lipid domains has been proposed to be an important regulatory step during the activation of the apoptotic death program (Lacour et al., 2004). Meanwhile, phosphoinositides such as phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate promote cell survival and protect against apoptosis by activating Akt/PKB, which phosphorylates components of the apoptotic machinery (Mejillano et al., 2001). Therefore, the regulation of membrane lipids levels in lipid rafts is one of the key factors of apoptotic signal in cells.

Cyclodextrins (CyDs) and their hydrophilic derivatives form inclusion complexes with hydrophobic molecules. CyDs can improve the solubility, dissolution rate and bioavailability of the drugs, and so the widespread use of CyDs is well known in the pharmaceutical field (Uekama et al., 1998). CyDs have been reported to interact with cell membrane constituents such as cholesterol and phospholipids (Motoyama et al., 2009b).

* Corresponding author at: Department of Physical Pharmaceutics, Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Chuo-ku, Kumamoto 862-0973, Japan, Tel.: +81 96 371 4160; fax: +81 96 371 4420.
E-mail address: arimah@gpo.kumamoto-u.ac.jp (H. Arima).

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Additionally, methyl-β-cyclodextrin (M-β-CyD) is acknowledged to disrupt the structures of lipid rafts and caveolae (Galbiati et al., 2001), which are lipid microdomains formed by lateral assemblies of cholesterol and sphingolipids in the cell membrane, through the extraction of cholesterol from the microdomains (Anderson and Jacobson, 2002). Furthermore, we demonstrated that 2,6-di-O-methyl-β-cyclodextrin (DM-β-CyD) induced apoptosis through the inhibition of PI3K-Akt-Bad pathway, leading to cholesterol depletion from lipid rafts in NR8383 cells, a rat alveolar macrophage cell line (Motoyama et al., 2009a). However, it is still not unclear whether M-β-CyD induces apoptosis or not through the interaction with membrane components in various tumor cells. Meanwhile, we previously revealed that the membrane interaction mode of M-β-CyD was somewhat different from that of DM-β-CyD (Motoyama et al., 2009a). Actually, M-β-CyD extracts cholesterol from cell membranes, while it interacts with phospholipids only very slightly. Meanwhile, DM-β-CyD has the potent cholesterol and phospholipids extraction ability from plasma membrane of cells. Due to the different interaction mode of M-β-CyD, compared to DM-β-CyD, against cell membrane components, in the present study, we therefore examined whether cell-death induced by M-β-CyD is apoptosis or not in various cells, and evaluated the potential of M-β-CyD as an antitumor agent *in vivo*.

2. Materials and methods

2.1. Materials

M-β-CyD with an average degree of substitution (DS) of methyl group of 12.2 was obtained from Junsei Chemical (Tokyo, Japan). RPMI-1640 culture medium (folic acid (FA)-free) was purchased from GIBCO (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Nissui Pharmaceuticals (Tokyo, Japan) and Nichirei (Tokyo, Japan), respectively. Tetramethylrhodamine isothiocyanate (TRITC) was obtained from Funakoshi (Tokyo, Japan). CellEvent™ Caspase-3/7 Green Detection Reagent and Cyto-ID™ Autophagy Detection Kit were purchased from Invitrogen (Tokyo, Japan) and Enzo Life Sciences (Farmingdale, NY), respectively. All other chemicals and solvents were of analytical reagent grade, and deionized double-distilled water was used throughout the study.

2.2. Cell culture

KB cells through referred to as a human oral squamous carcinoma cell line, which is now known to be a subline of the ubiquitous KERATIN-forming tumor cell line HeLa, were cultured as reported previously (Onodera et al., 2011). Ihara cells, a highly pigmented human melanoma cell line, and M213 cells, a human cholangiocarcinoma cell line, were grown in DMEM-high glucose containing glucose (4.5 mg/mL), penicillin (1×10^5 mU/mL) and streptomycin (0.1 mg/mL) supplemented with 10% FBS at 37 °C in a humidified 5% CO_2 and 95% air atmosphere.

2.3. In vitro cytotoxic activity

In vitro cytotoxic activity was assayed by the WST-1 method (a Cell Counting Kit, Wako Pure Chemical Industries, Osaka, Japan). Briefly, KB cells, Ihara cells, and M213 cells were seeded at 5×10^4 cells onto 24-well microplate (Iwaki, Tokyo, Japan) and incubated for 24 h in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Cells were washed twice with culture medium, and then incubated with 300 μL of culture medium containing 20 mM M-β-CyD in the presence or absence of cholesterol for 2 h at 37 °C. In the cholesterol-loading experiment, we used saturated cholesterol in culture medium obtained by 24 h after shaking with 5 mg of cholesterol and 20 mM M-β-CyD and filtering them. After washing twice with phosphate-buffered saline (PBS, pH 7.4) to remove M-β-CyD, 300 μL of fresh Hanks' balanced salt solution (HBSS, pH 7.4) and 30 μL of WST-1 reagent were added to the plates and incubated for 30 min at 37 °C. The absorbance at 450 nm against a reference wavelength of 630 nm was measured with a microplate reader (Bio-Rad Model 550, Tokyo, Japan).

2.4. Cellular association of M-β-CyD

KB cells, Ihara cells and M213 cells (1×10^6/35 mm dish) were incubated with 1 mL of culture medium containing 10 μM TRITC-labeled M-β-CyD (TRITC-M-β-CyD) at 37 °C for 1 h. After washing twice with 1 mL of PBS (pH 7.4) to remove the sample, and immediately scraped with 1 mL of PBS (pH 7.4). The cells were collected and filtered through nylon mesh. Data were collected for 1×10^4 cells on a FACSCalibur flow cytometer using CellQuest software (Becton-Dickinson, Mountain View, CA).

2.5. Intracellular distribution of M-β-CyD

KB cells (1×10^6/35 mm glass bottom dish) were incubated with 10 μM TRITC-M-β-CyD at 37 °C for 1 h. After incubation, Hoechst 33342 (10 μg/mL) was added to each well and incubated at 37 °C for 10 min. The cells were washed with RPMI-1640 culture medium (FA-free) twice, and were added 1 mL of RPMI-1640 culture medium (FA-free). The cells were observed by a fluorescence microscope (KEYENCE Biozero BZ-8000, Tokyo, Japan).

2.6. Determination of cholesterol in the culture medium

KB cells (1×10^6/35 mm dish) were incubated with 20 mM M-β-CyD in RPMI-1640 culture medium (FA-free) at 37 °C for 1 h. After centrifugation (10,000 rpm, 5 min) of the culture medium, the supernatant was recovered. Total cholesterol in the culture medium was determined using a Cholesterol-test Wako® (Wako Pure Chemical Industries, Osaka, Japan).

2.7. DNA content

For determination of DNA content in cells, KB cells (1×10^6/35 mm dish) were incubated with RPMI-1640 culture medium (FA-free) containing 20 mM M-β-CyD for 2 h. After washing with PBS, the cells were suspended and fixed with 500 μL of ice-cold 70% (v/v) ethanol for 4 h. After washing with PBS and subsequent centrifugation, cells were re-suspended with RNase A (100 μg/ml) and incubated for 30 min at 37 °C. After centrifugation, cells were resuspended in a solution containing 500 μL of propidium iodide (PI, 20 μg/mL) and incubated for 20 min on ice before quantification using a FACSCalibur flow cytometer with CellQuest software (Becton Dickinson, Mountain View, CA).

2.8. Mitochondrial transmembrane potential

To measure the mitochondrial transmembrane potential, rhodamine 123 was used as reported previously (Motoyama et al., 2009a). KB cells (1×10^6/35 mm dish) were incubated with RPMI-1640 culture medium (FA-free) containing 20 mM M-β-CyD for 2 h. After washed with PBS, cells were stained by 10 μM rhodamine 123 for 15 min at 37 °C. After washing once with 1 mL of PBS to remove the samples, cells were lysed by the addition of 1 mL of PBS. Then, mitochondrial transmembrane potential was analyzed by a FACSCalibur flow cytometer as described above.

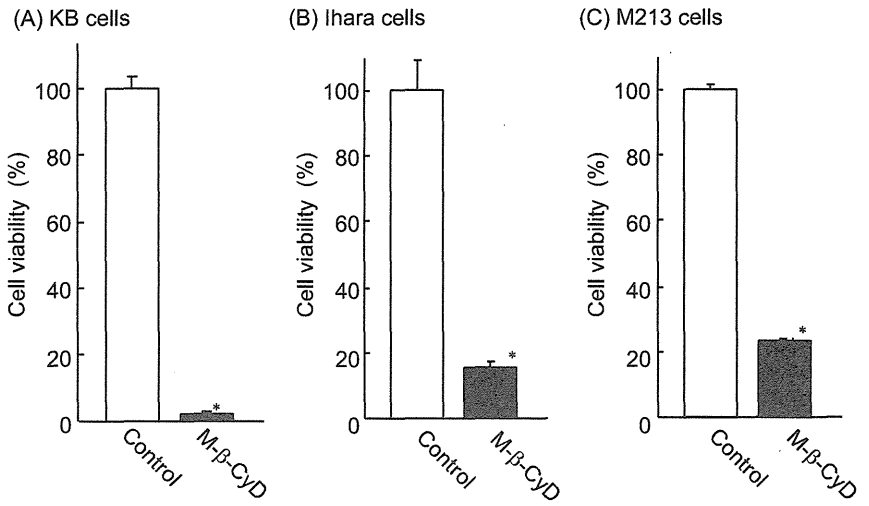

Fig. 1. Cytotoxic activity of M-β-CyD in various cells. (A) KB cells, (B) Ihara cells, and (C) M213 cells were incubated for 2 h with 300 μL of medium containing M-β-CyD (20 mM) at 37 °C. After washing twice with PBS to remove M-β-CyD, 300 μL of fresh HBSS and 30 μL of WST-1 reagent were added to plates and incubated for 30 min at 37 °C. Each value represents the mean ± S.E.M. of 3–4 experiments. $^*p < 0.05$, compared with control.

2.9. Activation of caspase-3/7

KB cells (1×10^6/35 mm dish) were incubated with 20 mM M-β-CyD for 2 h. After washing twice with 1 mL of RPMI-1640 (FA-free) medium to remove the samples, cells were add to 10 μM CellEvent™ Caspase-3/7 Green Detection Reagent (Invitrogen, Tokyo, Japan) and incubated at 37 °C for 30 min. The cells were washed with RPMI-1640 (FA-free) medium twice, and added 1 mL of RPMI-1640 (FA-free) medium. The cells were observed by a fluorescence microscope (KEYENCE Biozero BZ-8000, Tokyo, Japan).

2.10. Detection of autophagosome

Cyto-ID™ Autophagy Detection Kit was used for determination of autophagic vacuoles in cells using a novel dye that selectively labels autophagic vacuoles. Briefly, the cells (1×10^6/35 mm glass bottom dish) were incubated with RPMI-1640 culture medium for 24 h. After washing with PBS, the cells were incubated with 20 mM M-β-CyD for 2 h, and then the cells were treated with Cyto-ID™ Autophagy Detection Kit. After washing with RPMI-1640 culture medium, the cells were observed by a fluorescence microscope (KEYENCE Biozero BZ-8000, Tokyo, Japan).

2.11. Evaluation of antitumor effect of M-β-CyD

Four-weeks-old BALB/c male mice (*ca.* 20 g) were subcutaneously injected the suspension containing Colon-26 carcinoma cells (5×10^5 cells/100 μL), FR-α expressing cells on a hind leg. About 10 days later, the mannitol solution (5%) dissolved with M-β-CyD (10, 50, or 100 mg/kg) was administered by the single intratumoral injection to tumor bearing mice. The tumor volumes were determined by the equation (Volume = $LW^2/2$), where L is the longest dimension parallel to the skin surface and W is the dimension perpendicular to L and parallel to the surface. The body weight changes of tumor-bearing mice were monitored for 21 days. Animal experiments were approved by the Ethics Committee for Animal Care and Use of Kumamoto University (Approval ID: 24-286).

2.12. Data analysis

Data are given as the mean ± S.E.M. Statistical significance of mean coefficients for the studies was performed by analysis of variance followed by Scheffe's test. *p*-Values for significance were set at 0.05.

3. Results

3.1. Cytotoxic activity of M-β-CyD

To examine the cytotoxic activity of M-β-CyD, the cell viability of various tumor cells was determined by WST-1 method. In our previous study, the cytotoxic activity of 10 mM M-β-CyD for 2 h was somewhat weak. Therefore, 20 mM M-β-CyD was treated for 2 h in cytotoxic study. M-β-CyD had potent cytotoxic activity, compared to control, in KB cells, Ihara cells and M213 cells, after treatment for 2 h (Fig. 1A–C). These results suggest that M-β-CyD had potent cytotoxic activity.

3.2. Cellular association and intracellular distribution of M-β-CyD

To gain insight into the mechanism for the cytotoxic activity of M-β-CyD, we examined the cellular association of TRITC-M-β-CyD after treatment for 1 h with various cells (Fig. 2). Herein the incubation time of M-β-CyD was set for 1 h, because KB cells could not survive for more than 2 h. TRITC-M-β-CyD associated with neither KB cells, Ihara cells, nor M213 cells (Fig. 2B–D). Additionally, we investigated the intracellular distribution of TRITC-M-β-CyD in KB cells after 1 h treatment using a fluorescent microscope (Fig. 2E). In consistent with the results of Fig. 2B, cellular uptake of TRITC-M-β-CyD in KB cells was not observed, compared to that of rhodamin123 as a positive control (Fig. 2A). Collectively, these results suggest that M-β-CyD was biomembrane-impermeable.

3.3. Effect of cholesterol extraction ability on cytotoxic activity of M-β-CyD

Lipid rafts are mainly composed of cholesterol and sphingolipids in the cell membranes, and contain various signal transduction molecules including growth factor receptors (Le Roy and Wrana, 2005). We previously reported that CyDs showed hemolytic activity at high concentration through the extraction of cell membrane components such as cholesterol and phospholipids from lipid rafts (Motoyama et al., 2006; Ohtani et al., 1989). Furthermore, we

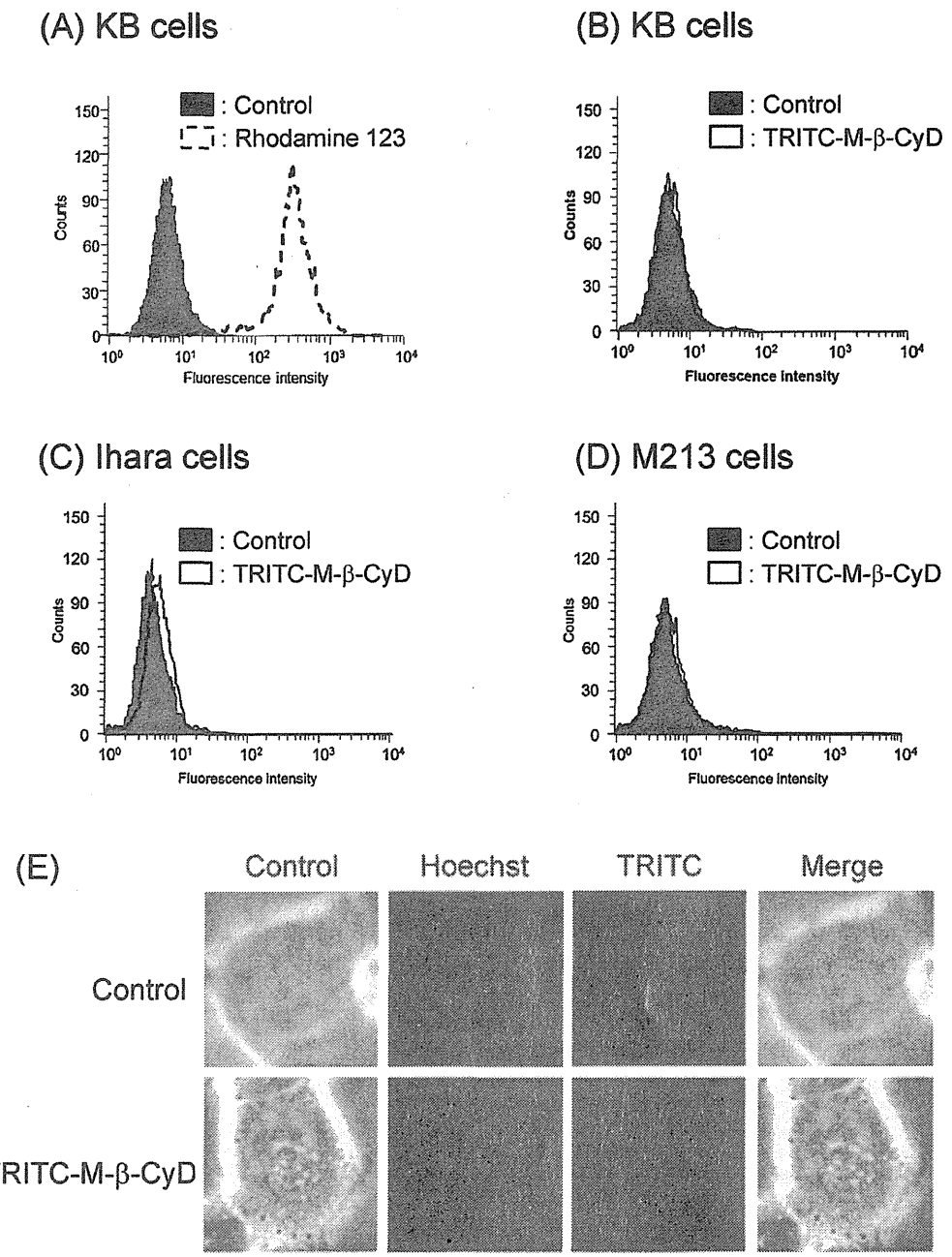

Fig. 2. Cellular association of TRITC-M-β-CyD in various cells. The fluorescence intensities of (A) rhodamine 123 in KB cells and TRITC in (B) KB cells, (C) Ihara cells, and (D) M213 cells were determined 1 h after incubation at 37 °C by a flow cytometer. (E) Intracellular distribution of TRITC-M-β-CyD in KB cells after treatment for 1 h. The experiments were performed independently three times, and representative data are shown.

demonstrated that DM-β-CyD induces apoptosis through cholesterol depletion in NR8383 cells (Motoyama et al., 2009a). Therefore, to reveal whether cell-death induced by M-β-CyD is apoptosis through cholesterol depletion in tumor cells, we investigated the effects of M-β-CyD on the release of cholesterol from KB cells to culture medium. Cholesterol released in the culture medium after incubation with 20 mM M-β-CyD for 1 h was determined by Cholesterol-test Wako®. The extent of cholesterol efflux by M-β-CyD was significantly higher than that of control in KB cells (Fig. 3).

Next, to confirm the involvement of cholesterol depletion in cell-death induced by M-β-CyD, we examined the cell viability of KB cells in the cholesterol-loaded medium containing M-β-CyD. Importantly, the cytotoxic activity of M-β-CyD in KB cells was significantly lowered in saturated cholesterol-loading culture medium (Fig. 4). Taken together, these results suggest that the extraction ability of M-β-CyD on cholesterol from plasma membranes may be associated with the cytotoxic activity in KB cells.

3.4. M-β-CyD caused cell-death in apoptosis-dependent pathway

To investigate whether M-β-CyD-induced cell-death is accompanied by apoptotic feature, we next examined the DNA content in nucleus, mitochondrial transmembrane potential, and caspase-3/7 activity in KB cells (Fig. 5). The DNA content in nucleus after incubation with 20 mM M-β-CyD for 2 h was strikingly decreased compared to that with control (without CyD) in KB cells (Fig. 5A).

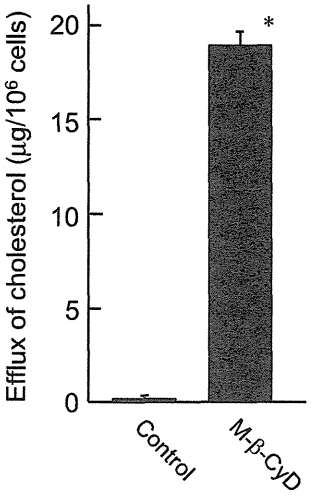

Fig. 3. Efflux of cholesterol from lipid rafts to culture medium after treatment with M-β-CyD (20 mM) in KB cells. Each value represents the mean ± S.E.M. of 3–4 experiments. *$p < 0.05$, compared with control.

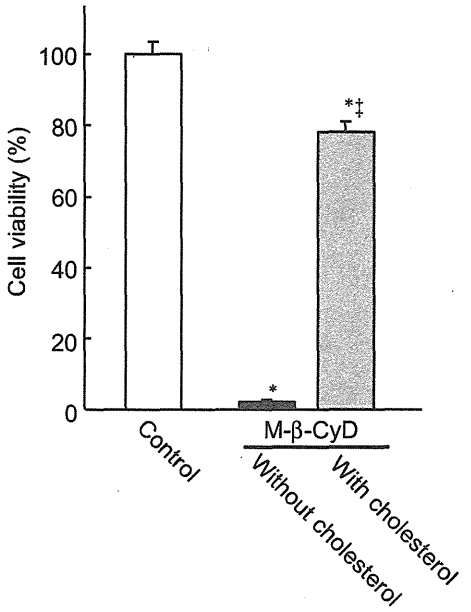

Fig. 4. Effect of cholesterol on cytotoxic activity of M-β-CyD (20 mM) in KB cells. Each value represents the mean ± S.E.M. of 6–9 experiments. *$p < 0.05$, compared with control. ‡$p < 0.05$, compared with M-β-CyD without cholesterol.

Next, we studied the effects of CyDs on mitochondrial transmembrane potential using rhodamine 123 in KB cells (Fig. 5B). The mitochondrial transmembrane potential of KB cells treated with M-β-CyD was significantly decreased, compared to that with control (without CyD).

Activation of caspase-3/7 is considered an essential event during apoptosis. We next examined the caspase-3/7 activity in KB cells after treatment with M-β-CyD using the CellEvent™ Caspase-3/7 Green Detection Reagent (Fig. 5C). This caspase-3/7 detection reagent is intrinsically non-fluorescent as the DEVD peptide inhibits the ability of the dye to bind to DNA. However, after activation of caspase-3/7 in apoptotic cells, the DEVD peptide is cleaved and enabled the dye to bind to DNA and produce a bright, fluorogenic response. Treatment of KB cells with M-β-CyD

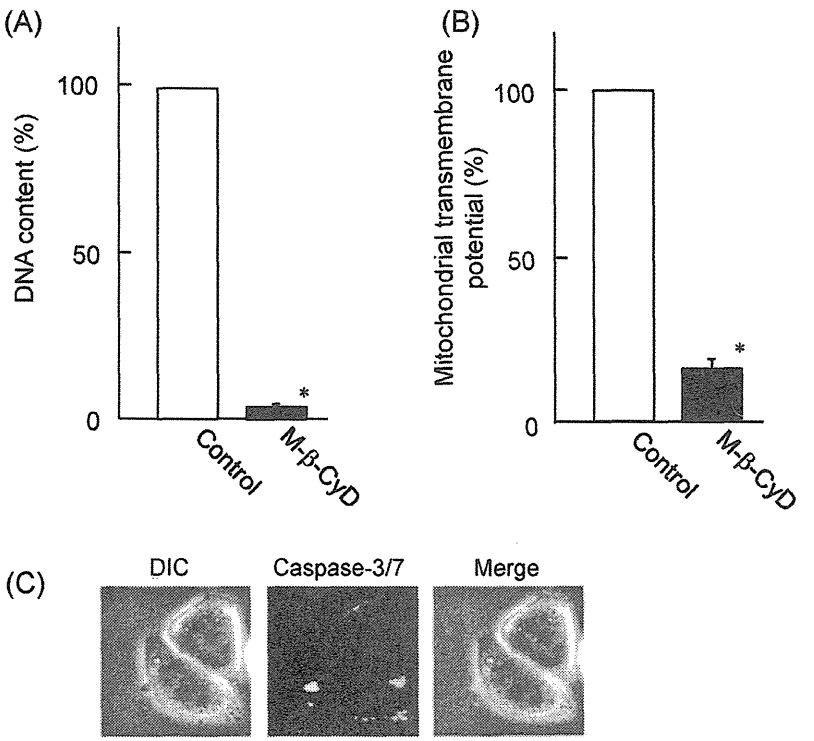

Fig. 5. Induction of apoptosis, but not autophagy, in KB cells after treatment with M-β-CyD. Effects of M-β-CyD on DNA content (A) and mitochondrial transmembrane potential (B). KB cells were treated with M-β-CyD (20 mM) for 2 h. Each value represents the mean ± S.E.M. of 3–4 experiments. *$p < 0.05$, compared with control. (C) Caspase-3/7 activity in KB cells after treatment with M-β-CyD. The experiments were performed independently three times, and representative images are shown.

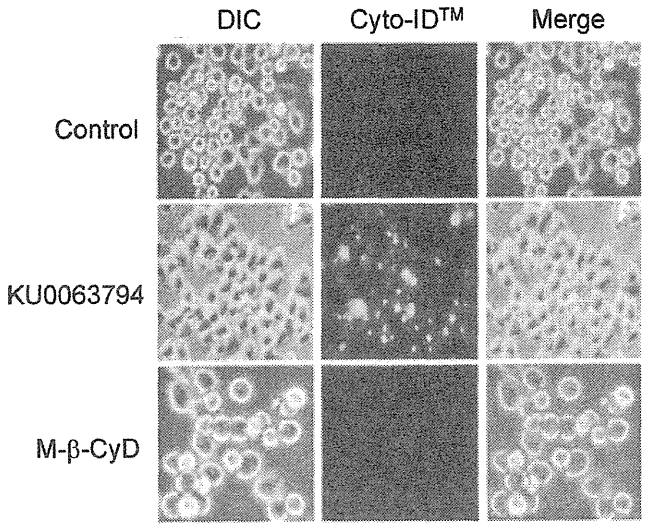

Fig. 6. Effects of M-β-CyD on the formation of autophagosomes in KB cells. KB cells were treated with M-β-CyD (20 mM) for 2 h, and then the cells were treated with Cyto-ID™. The concentration of KU0063794, an autophagy inducer, was 10 μM. After washed twice with PRMI medium (FA (+)), cells were scanned with a fluorescence microscope. The experiments were performed independently three times, and representative images are shown.

for 2 h caused caspase-3/7 activation (Fig. 5C). Collectively, these results strongly suggest that M-β-CyD caused cell-death in KB cells in apoptosis-dependent pathway.

Autophagy can promote cell adaptation and survival, but under some conditions it leads to cell-death. A number of studies have reported that autophagy or autophagic cell-death is activated in cancer cells that are derived from tissues such as breast, colon, prostate and brain, in response to various anticancer therapies (Kondo et al., 2005). Therefore, we investigated whether M-β-CyD induces autophagic cell-death in KB cells or not, using Cyto-ID™ Autophagy Detection Kit, which measures autophagic vacuoles such as pre-autophagosomes, autophagosomes, and autolysosomes (autophagolysosomes) in live cells. As shown in Fig. 6, the autophagic vacuoles in KB cells did not observe after treatment with M-β-CyD for 2 h. These results suggest that M-β-CyD did not induce autophagic cell-death in KB cells.

3.5. Antitumor activity of M-β-CyD in tumor-bearing mice

To investigate the antitumor activity of M-β-CyD *in vivo*, we injected M-β-CyD solution intratumorally to Colon-26 cells-bearing mice. Here we selected Colon-26 cells for *in vivo* experiments, because the cells express FR-α and are often used as allografted tumor model. As shown in Fig. 7A, an intratumoral injection of the M-β-CyD at a dose of 10, 50, and 100 mg/kg drastically inhibited the tumor growth, compared to that of control (5% mannitol solution). In the viewpoint of safety, the body weight of mice after an intratumoral injection of M-β-CyD was slightly increased as the time passed (Fig. 7B). These results suggest that M-β-CyD had the potent antitumor activity after intratumoral injection to tumor-bearing mice.

4. Discussion

In the present study, we revealed that M-β-CyD potentially caused apoptosis in KB, Ihara and M213 cells, possibly due to cholesterol depletion from lipid rafts, and M-β-CyD drastically inhibited the tumor growth after intratumoral injection to Colon-26 cells-bearing mice.

Generally, it is difficult for CyDs to induce apoptosis after uptake into cells, because CyDs have poor membrane permeability due to its aqueous properties and high molecular weight. Rosenbaum et al. reported, however, that β-CyD/dextran conjugates can enter cells and located cholesterol-enriched lysosomal storage organelles, and were effective at reducing the cholesterol accumulation (Rosenbaum et al., 2010). Additionally, Plazzo et al. also reported that M-β-CyD tagged with fluorescein can be internalized *via* clathrin-dependent endocytosis in HeLa cells. To resolve this controversial, we examined the cellular association of TRITC-M-β-CyD in various cells. As a result, TRITC-M-β-CyD was associated with KB cells, Ihara cell, and M213 cells only very slightly under the present experimental conditions (Fig. 2B–D). Therefore, we hypothesized that apoptosis induced by CyDs is involved in the interaction with plasma membrane components. Actually, non-ionic surfactants, such as polyethylene glycol sorbitan monolaurate (Tween 20), polyoxyethylenehydrogenated caster oil 60 (HCO-60) and Triton X-100, induced apoptosis in NR8383 cells at more than critical micelle concentrations, probably due to the solubilizing effects of cell membrane components such as cholesterol and phospholipids (data not shown). Therefore, it is highly likely that the solubilizing effects of M-β-CyD on membrane lipids resulted in the

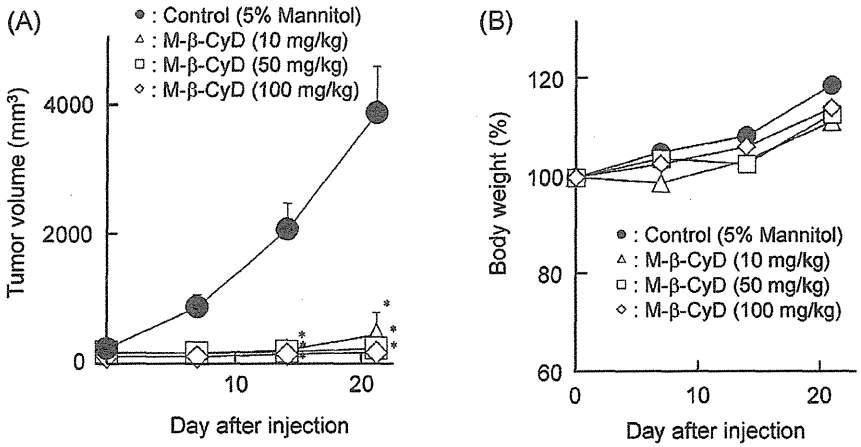

Fig. 7. Effects of intratumoral administration of M-β-CyD on tumor growth (A) and body weight (B) in tumor-bearing mice. The dose of M-β-CyD was 10, 50 or 100 mg/kg. The number of mice for Control, M-β-CyD (10 mg/mL), M-β-CyD (50 mg/mL) and M-β-CyD (100 mg/mL) was 5, 3, 3 and 3, respectively. *$p < 0.05$, compared with control (5% mannitol solution).

induction of apoptosis, because M-β-CyD has high hemolytic activity through the solubilizing effects on cholesterol (Irie and Uekama, 1997, 1999).

Recently, it is reported that lipid rafts, which are lipid microdomains mainly composed of cholesterol and sphingolipids, are contributed to apoptosis *via* FasL/Fas and Bad, an apoptosis inducible factor of Bcl-2 family (Ayllon et al., 2002; Hueber et al., 2002; Legler et al., 2003). In addition, we previously revealed that M-β-CyD extracted cholesterol from lipid microdomains from rabbit red blood cells (Motoyama et al., 2009b). Therefore, we examined whether apoptosis induced by M-β-CyD is contributed to the extraction of cholesterol from lipid rafts. Actually, M-β-CyD significantly released cholesterol from lipid rafts into the culture medium in KB cells (Fig. 3). Moreover, the cytotoxic activity of M-β-CyD was potentially inhibited by the addition of cholesterol in culture medium (Fig. 4). Therefore, these results strongly suggest that cholesterol depletion in lipid rafts had a crucial role in the induction of apoptosis by M-β-CyD. Further elaborate studies on not only the extent of cholesterol after treated with M-β-CyD in cell membranes, but also the relation of extracted cholesterol and cell death should be performed.

PI3K activates Akt through the recruitment of Akt and PDK1 (3-phospho-inositide-dependent protein kinase-1) to lipid rafts caused by production of PI(3,4,5)P3. This activated-Akt by PI3K suppresses the induction of apoptosis through the phosphorylation of Bad and caspase-9. Previously, we demonstrated that DM-β-CyD significantly suppressed phosphorylation of Akt and accelerated degradation of Akt in NR8383 cells through the extraction of cholesterol from lipid rafts (Motoyama et al., 2009a). Furthermore, we revealed that DM-β-CyD potentially suppressed phosphorylation of Bad (Motoyama et al., 2009a). Zha et al. reported that suppression of Bad phosphorylation binds with Bcl-xL, an apoptosis inhibitory factor on mitochondrial cell membrane, resulting in induction of apoptosis (Zha et al., 1996). Therefore, it can be thought that M-β-CyD could suppress the activation of PI3K-Akt-Bad pathway, as well as DM-β-CyD, in apoptotic cell-death induction.

Signal transduction regarding the induction of apoptosis is largely discriminated into a mitochondrial-dependent or -independent pathway. Mitochondrion have crucial roles in signal transduction of apoptosis, because the activation of caspase family is induced through the reduction of mitochondrial transmembrane potential following the release of cytochrome *c* prior to apoptosis. To release cytochrome *c* from mitochondria during apoptosis, the formation of VDAC (voltage-dependent anion channel) tetramer on the mitochondrial membrane plays an important role (Shimizu et al., 2001, 1999). On the other hand, Bcl-xL regulates the release of cytochrome *c* through the suppression of VDAC channel activity (Yang et al., 1995; Zha et al., 1996). As apoptosis induction by Bad is contributed to the binding of Bad with Bcl-xL on the mitochondrial membrane. Previously, we revealed that the inhibition of phosphorylation of Bad by DM-β-CyD induced the collapse of mitochondrial transmembrane potential and released cytochrome *c* from mitochondria (Motoyama et al., 2009a). In the present study, we demonstrated that M-β-CyD lowered the mitochondrial transmembrane potential in KB cells (Fig. 5B). Therefore, apoptosis induced by M-β-CyD was found to be potentially involved in the mitochondria-dependent pathway. In addition to fluorescence staining (Fig. 5C), in order to further confirm the caspase 3/7 activation in KB cells after treatment with M-β-CyD, western blot analysis is thereafter required.

It should be noted that M-β-CyD provided *in vivo* antitumor activity after an intratumoral injection to Colon-26 cells-bearing mice (Fig. 7). Grosse et al. reported that the antiproliferative activity of M-β-CyD was statistically higher than that of doxorubicin after intraperitoneal administrations to Swiss nude mice xenografted with MCF7 or A2789 tumor cells (Grosse et al., 1998). Thus, M-β-CyD is likely to be more preferable to doxorubicin from the viewpoint of both pharmacological effects. However, an intravenous administration of M-β-CyD did not show any significant antitumor activity (data not shown), probably due to the lack of target specificity against tumor cells and rapid renal clearance from body. Therefore, we are currently investigating the *in vitro* and *in vivo* antitumor activity of M-β-CyD modified with folic acid as a tumor targeting ligand, which recognizes by folate receptor-α-overexpressing tumor cells.

In conclusion, the present study may demonstrate that M-β-CyD induced mitochondria-dependent apoptosis through cholesterol depletion in lipid rafts of tumor cells, and had the potential of a novel antitumor agent and/or its lead compound.

Acknowledgements

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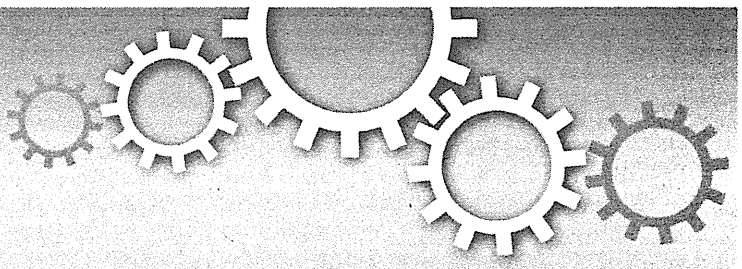

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Correspondence and requests for materials should be addressed to H.A. (arimah@gpo.kumamoto-u.ac.jp)

Involvement of Autophagy in Antitumor Activity of Folate-appended Methyl-β-cyclodextrin

Risako Onodera[1], Keiichi Motoyama[1], Nao Tanaka[1], Ayumu Ohyama[1,3], Ayaka Okamatsu[1], Taishi Higashi[1], Ryusho Kariya[2], Seiji Okada[2] & Hidetoshi Arima[1,3]

[1]Department of Physical Pharmaceutics, Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Chuo-ku, Kumamoto 862-0973, Japan, [2]Division of Hematopoiesis, Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Chuo-ku, Kumamoto 860-0811, Japan, [3]Program for Leading Graduate Schools "HIGO (Health life science: Interdisciplinary and Glocal Oriented) Program", Kumamoto University.

Autophagy, the major lysosomal pathway for recycling intracellular components including organelles, is emerging as a key process regulating tumorigenesis and cancer therapy. Most recently, we newly synthesized folate-appended methyl-β-cyclodextrin (FA-M-β-CyD), and demonstrated the potential of FA-M-β-CyD as a new antitumor drug. In this study, we investigated whether anticancer activity of FA-M-β-CyD in folate receptor-α (FR-α)-positive tumor cells is involved in autophagy. In contrast to methyl-β-cyclodextrin (M-β-CyD), FA-M-β-CyD entered KB cells (FR-α (+)) through CLIC/GEEC endocytosis. No significant depression in the DNA content was observed in KB cells after treatment with FA-M-β-CyD. Additionally, the transmembrane potential of mitochondria after treatment with FA-M-β-CyD was drastically elevated. Meanwhile, FA-M-β-CyD induced the formation of autophagic vacuoles, which were partially colocalized with mitochondria, in KB cells. Taken together, these results suggest that FR-α-expressing cell-selective cytotoxic activity of FA-M-β-CyD could be mediated by the regulation of autophagy, rather than the induction of apoptosis.

In cancer chemotherapy, to obtain the maximum treatment efficacy of anticancer agents, the drug delivery technique is extremely important. To confer an active targeting-ability, the chemical modification of tumor-specific ligands to a drug carrier is known. Of various tumor-specific ligands, folic acid (FA)[1–5] has emerged as a remarkable targeting ligand capable of potent interaction with cancer cells expressing the folate receptor (FR) with high affinity (K_d: $10^{-9} \sim 10^{-10}$ M)[6,7]. FR is engaged the cell surface through a glycosylphosphatidylinositol-anchor, and is highly expressed in various tumor cells including malignancies of the brain, ovary, breast, kidney, and lung, and has negligible expression in normal tissues[8]. In addition, as a cancer progress, the expression level of FR increase remarkably[9]. Therefore, FR is one of the potent candidate for not only a promising marker but also a target protein for therapy of cancer.

Cyclodextrins (CyDs) are cyclic oligosaccharides forming inclusion complexes with a wide range of hydrophobic molecules, and are used widely in pharmaceutical region[10,11]. CyDs have been reported to interact with cell membrane components of cholesterol and phospholipids, resulting in the induction of hemolysis of red blood cells at high concentrations of CyDs[12–14]. In Addition, methyl-β-cyclodextrin (M-β-CyD) is often used to disrupt lipid rafts because of its ability to decrease cholesterol stores on cell membranes[15]. A number of studies have also demonstrated that the disruption of lipid rafts by M-β-CyD can harm cancer cells and cause cell-death. Notedly, Grosse *et al.* revealed that M-β-CyD significantly reduced tumor growth in tumor-bearing mice after intraperitoneal administration[16]. However, the cytotoxic reaction of M-β-CyD has a lack of a tumor cell-selectivity.

Most recently, to make an attempt to give a tumor-specific cytotoxic reaction to M-β-CyD, we previously prepared FA-conjugated M-β-CyD (FA-M-β-CyD)[17], and evaluated its antitumor activity[18]. FA-M-β-CyD provided great antitumor activity, compared to M-β-CyD in KB cells, highly expressing folate receptor-α (FR-α). The single intravenous administration of FA-M-β-CyD significantly suppressed the tumor growth in Colon-26 cells (FR-α (+))-bearing mice. Additionally, the antitumor activity of FA-M-β-CyD was superior to that of doxorubicin after an intravenous administration, at the same dose. These results indicate that FA-M-β-CyD has the potential as a promising anticancer agent. However, the mechanism of antitumor activity of FA-M-β-CyD still remains unclear.

Autophagy, the major lysosomal pathway for recycling intracellular components including organelles, is emerging as a key process regulating tumorigenesis and cancer therapy[19–23]. The dynamic roles for autophagy in cancer is tumor suppressive effect in the early stage of cancer development, but is the growth effect of established tumors. Likewise, the stimulation of autophagy in response to therapeutics can contextually favor or weaken chemoresistance and antitumor immunity. Therefore, the understanding whether and how autophagy can be harnessed to kill cancer cells is essential for cancer chemotherapy. In this study, we investigated whether antitumor activity of FA-M-β-CyD in FR-α (+) cells is involved in autophagy. As a result, FA-M-β-CyD was found to induce autophagosome formation in FR-α (+) cells, indicating the involvement of autophagy in antitumor activity. Taking into the consideration of our previous results that FA-M-β-CyD drastically suppressed the tumor growth in mice inoculated FR-α (+) tumor cells[18], FA-M-β-CyD can be applied as a novel anticancer drug through regulating autophagy for cancer chemotherapy against FR-α-overexpressing tumor.

Results

Antitumor effect of FA-M-β-CyD. To elucidate the antitumor effect of FA-M-β-CyD in FR-α (+) cells, we investigated antitumor effect of FA-M-β-CyD in FR-α (+) and FR-α (−) cells. FA-M-β-CyD had great antitumor effect in FR-α (+) cells such as KB and M213 cells, compared to control, after treatment for 2 h (Fig. 1A, B). However, there was no significant antitumor activity in FR-α-negative A549 cells (Fig. 1C). These results indicate that FA-M-β-CyD had FR-α (+) cell-selective antitumor effect.

Cellular association and intracellular distribution of FA-M-β-CyD. Previously, we reported that FA-M-β-CyD possesses FR-α (+) cell-specific antitumor effect[18]. To obtain the detail of the mechanism for the FR-α-mediated antitumor effect of FA-M-β-CyD, we studied whether TRITC-FA-M-β-CyD associates with KB cells (Fig. 2). Strikingly, TRITC-FA-M-β-CyD highly associated with KB cells (Fig. 2A), despite CyDs are known to be biomembrane-impermeable. Furthermore, the association of TRITC-FA-M-β-CyD was inhibited by the addition of FA as a competitor of FR (Fig. 2A). Similar results were observed in M213 cells (Fig. 2B). Additionally, cellular association of FA-M-β-CyD was significantly lowered in FR-α down-regulated KB cells, compared to KB cells (Fig. 2C). These data indicate that FA-M-β-CyD could associate with cells through FR-α.

Next, we examined the intracellular distribution of TRITC-FA-M-β-CyD in KB cells after 1 h treatment (Fig. 3). It should be noted that cellular uptake of TRITC-FA-M-β-CyD in KB cells was observed. Furthermore, TRITC-FA-M-β-CyD mainly localized in cytoplasm rather than in nucleus after 1 h treatment. Collectively, these results indicate that FA-M-β-CyD distributed in cytoplasm after the cellular uptake into KB cells, and provided potent antitumor effects.

FA-M-β-CyD caused cytotoxic activity via apoptosis-independent pathway. To examine whether FA-M-β-CyD induces apoptosis or not, we investigated the DNA content in nucleus, transmembrane potential in mitochondria, TUNEL assay, and caspase 3 cleavage assay in KB cells. Here, we used 2, 6-di-*O*-methyl-β-CyD (DM-β-CyD) as a positive control of apoptosis inducer, because we previously demonstrated that DM-β-CyD elicited apoptosis through the suppression of the PI3K-Akt activity, through cholesterol extraction from plasma membranes in NR8383 cells[24]. The DNA content in KB cells after treatment with 10 mM DM-β-CyD for 2 h was significantly lowered, compared to that with control (Fig. 4A). Meanwhile, no significant decrease in the DNA content was observed in 10 mM FA-M-β-CyD in KB cells (Fig. 4A).

Next, we investigated the effects of CyDs on transmembrane potential in mitochondria using rhodamine 123 in KB cells (Fig. 4B). The transmembrane potential in mitochondria of KB cells

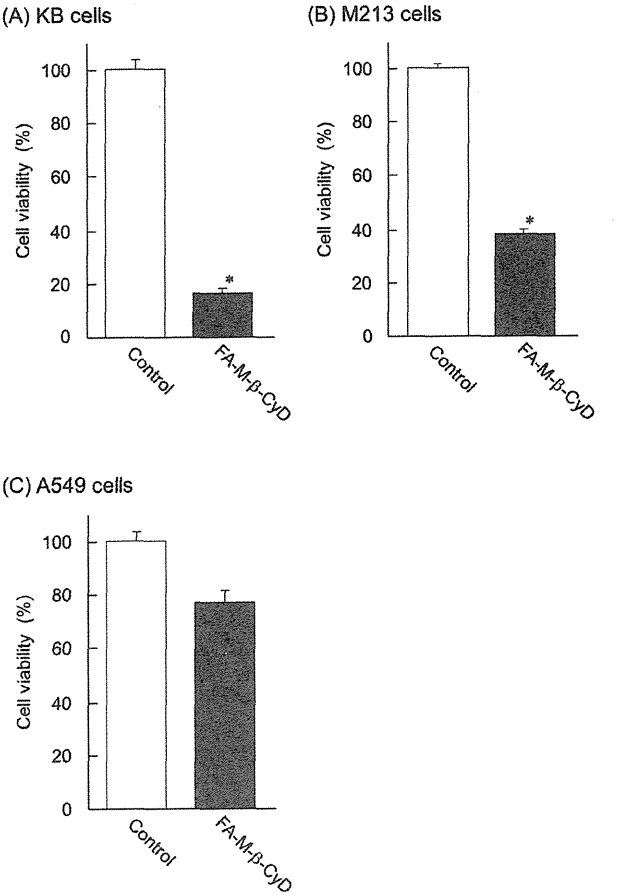

Figure 1 | Antitumor effect of FA-M-β-CyD. (A) KB cells, (B) M213 cells, and (C) A549 cells. The concentration of FA-M-β-CyD was 10 mM. Results are represented as mean ± S.E.M. (n = 3–4 per group). $*p < 0.05$ vs. control.

treated with DM-β-CyD was drastically decreased, compared to control. In sharp contrast, the potential of KB cells treated with FA-M-β-CyD was drastically elevated. Furthermore, this increment of the potential by the addition of FA-M-β-CyD was decreased to control level in the presence of FA, a competitor of FR (Fig. 4B).

Next, we performed TUNEL assay. As shown in Fig. 4C, KB cells treated with DM-β-CyD were stained, compared to control, suggesting the induction of apoptosis. Meanwhile, the cells treated with FA-M-β-CyD were not stained.

Furthermore, in the cleaved caspase 3 assay (Fig. 4D, 4E), DM-β-CyD potently produced activated-caspase 3 through a cleavage of pro-caspase 3, indicating the induction of apoptosis. However, FA-M-β-CyD showed only slight cleavage activity for pro-caspase 3. Collectively, these data indicate that cell-death caused by FA-M-β-CyD was apoptosis-independent.

Involvement of autophagy in cell-death caused by FA-M-β-CyD. Autophagy is a normal physiological process in the body that deals with destruction of cells in the body, and can kill the cells under certain conditions. There are several reports on autophagy or autophagic cell-death activated in cancer cells after treatment with various anticancer drugs[25]. Next, we examined whether autophagosome formation in KB cells is elicited by FA-M-β-CyD, using Cyto-ID® Autophagy Detection Kit, which detects autophagic vacuoles in cells. As shown in Fig. 5A and 5B, the autophagic

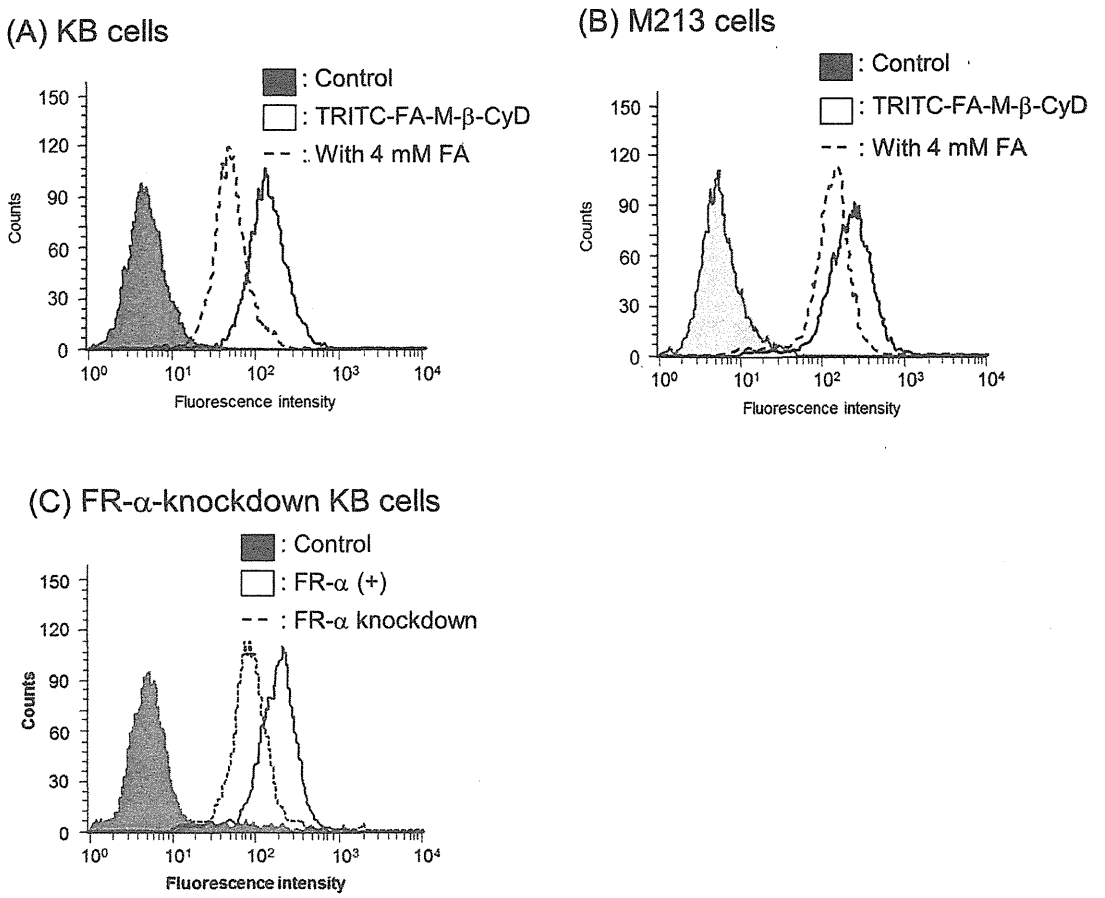

Figure 2 | Cellular association of TRITC-FA-M-β-CyD. (A) KB cells, (B) M213 cells and (C) FR-α-knockdown cells. The fluorescence intensity derived from TRITC was determined 1 h after incubation at 37°C by a flow cytometer.

vacuoles in KB cells were observed after treatment with FA-M-β-CyD for 2 h. Additionally, the autophagic vacuoles elicited by the treatment with FA-M-β-CyD were overwhelmingly decreased by the pretreatment of LY294002, an autophagy inhibitor. These results suggest that FA-M-β-CyD induced the formation of autophagic vacuoles in KB cells.

Next, we performed autophagy assay using a kit of Premo™ Autophagy Sensors, which can monitor a clearance of protein aggregates via autophagy using GFP-labeled p62 in Fig. 5C and 5D. Here, the p62 protein is able to bind to both ubiquitin[26] and LC3[27], thereby facilitating clearance of ubiquitinated proteins via autophagy. The fluorescence of GFP-p62 in control was drastically lowered by the addition of FA-M-β-CyD. Meanwhile, M-β-CyD did not show significant change in the fluorescence of GFP-p62, compared to control. These results suggest that the accumulated autophagosomes in KB cells were degraded by FA-M-β-CyD via autophagy.

Next, we examined the effects of autophagy inhibitors such as chloroquine, bafilomycin A1, 3-methyladenine (3-MA), and LY294002 on cell viability of KB cells after treatment with FA-M-β-CyD. Here, chloroquine and bafilomycin A1 prevent endosomal acidification, which leads to inhibition of both fusion of autophagosome with lysosome and lysosomal protein degradation. 3-MA and

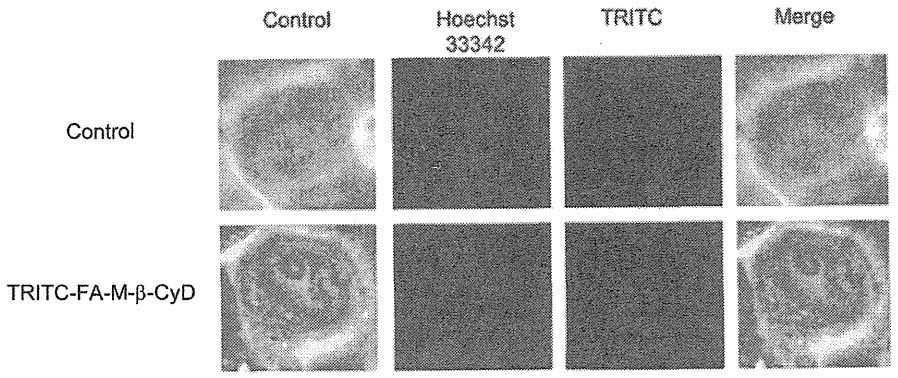

Figure 3 | Intracellular distribution of TRITC-FA-M-β-CyD. KB cells were treated with TRITC-FA-M-β-CyD (10 μM) for 1 h.

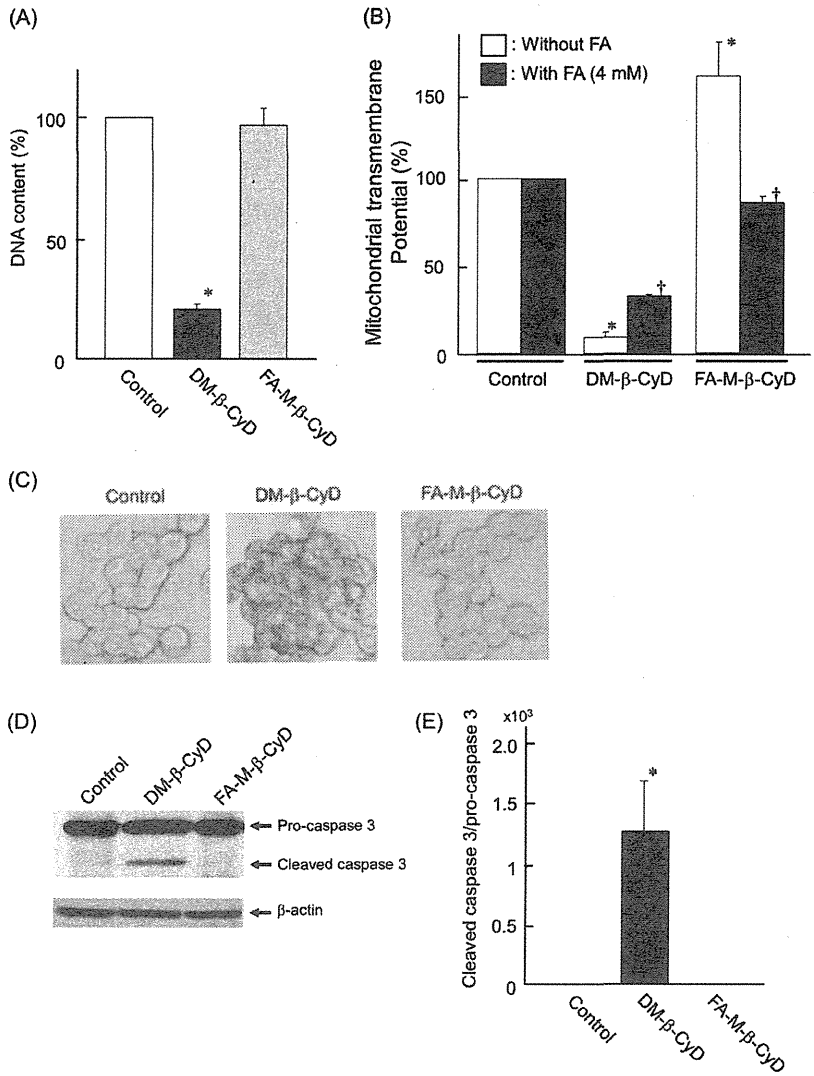

Figure 4 | DNA content (A) and mitochondrial transmembrane potential (B) after treatment with FA-M-β-CyD. KB cells were treated with FA-M-β-CyD (10 mM) for 2 h. Results are represented as mean ± S.E.M. (n = 3–4 per group). $*p < 0.05$ vs. control. $\dagger p < 0.05$ vs. KB cells without treatment of FA. (C) TUNEL assay after treatment with FA-M-β-CyD in KB cells. (D) Cleaved caspase 3 assay after treatment with FA-M-β-CyD in KB cells. The cropped blots were indicated. (E) The band intensity of cleaved caspase 3/pro-caspase 3 ratio. Results are represented as mean ± S.E.M. (n = 3 per group). $*p < 0.05$ vs. control.

LY294002 were used as PI3K inhibitors. As shown in Fig. 5E, the cell viability of KB cells treated with FA-M-β-CyD in the presence of autophagy inhibitors was higher than that with FA-M-β-CyD alone. Taken together, these data indicate that FA-M-β-CyD is likely to cause autophagic cell-death.

The dysfunctional mitochondria are recognized and degraded within cells by both non-selective autophagy and mitophagy, a selective type of autophagy[28,29]. As shown in Fig. 4B, we found that FA-M-β-CyD significantly enhanced the mitochondrial membrane potential in KB cells, indicating the induction of mitochondrial stress. Therefore, we examined the involvement of mitophagy in cell-death caused by mitochondrial stress after treatment with FA-M-β-CyD (Fig. 6). The autophagic vacuoles and mitochondria, stained by Cyto-ID® Autophagy Detection Kit and rhodamine 123, respectively, were partially colocalized in KB cells after treatment with FA-M-β-CyD (Fig. 6A). Similar results were obtained in M213 cells (Fig. 6B). Therefore, these results suggest that the autophagic cell-death induced by FA-M-β-CyD could be associated with mitophagy elicited by a mitochondrial stress.

Discussion

Having a targeting ability of antitumor agents plays a key role to not only provide strong antitumor activity but also reduce a risk of side effects in cancer chemotherapy. Previously, we demonstrated that FA-M-β-CyD showed a FR-α (+) cell-selective antitumor effect[17]. Additionally, we revealed that the antitumor effect of FA-M-β-CyD was significantly suppressed in the presence of FA, indicating that FR-mediated endocytosis is crucial for the enhancement of antitumor effect by FA-M-β-CyD[18]. In generally, it is believed that the extent of cellular uptake of CyDs is negligible probably due to their hydrophilicity and high molecular weight, FA-M-β-CyD was actually internalized into KB cells (Fig. 3). Meanwhile, FR was thought to be endocytosed via clathrin-independent carrier/GPI-anchored proteins enriched early endosomal compartment (CLIC/GEEC)[30]. Therefore, FA-M-β-CyD could enter the cells via CLIC/GEEC after the recognition by FR-α. Actually, FA-M-β-CyD highly associated with KB cells rather than that with FR-α-knockdown KB cells (Fig. 2C), indicating the potential of FA-M-β-CyD as a FR-α (+) cell-selective anticancer drug.