抗体はC型肝炎治療の様々な局面における応用が期待される．

5 TJを標的とした創薬研究の課題

上述したように，上皮細胞生物学の進展により，TJを標的とした創薬研究は薬物吸収促進において質的な発展を遂げ，がんや感染症などへの展開も拓けつつある．本稿の最後に，TJを標的とした創薬研究の課題や展望について触れてみたい．

TJシール制御による薬物送達では，細胞間隙経路の開口に伴う非特異的な物質の流入が実用化に向けた課題となっている．クローディンは27種類存在するメンバーの多種多様な組み合わせにより生体内の多様な内部環境維持に関与していると考えられており，クローディンバリアを自由自在に制御する技術を構築することで透過物質特異的な薬物送達法の開発が可能になると期待されている．しかしながら，いまだ本POCは確立されておらず，クローディンバリア制御の安全性評価が焦眉の急となっている．

また，クローディンを標的としたがん・ワクチン・C型肝炎治療法の顕在化では，クローディン結合分子の創製が成否を握っているものの，クローディンは細胞外領域が小さいうえにタンパク質精製が困難であり(現在のところ，claudin-4しか精製系が確立されていない)，抗体を含めてクローディン結合分子の創製は立ち遅れている．ようやく最近，国内外の複数のグループにおいて，*claudin*遺伝子を免疫，クローディン発現細胞を免疫することでクローディンの細胞外領域を認識するモノクローナル抗体が創出されており，クローディン結合分子創製技術が確立されつつある．[18,22] さらに，東京大学先端科学技術センター・浜窪隆雄博士が開発した発芽バキュロウイルス(BV)を利用した膜タンパク質発現系を用い，クローディン提示BVがクローディン結合分子スクリーニング系として機能することも見いだされており，抗体作製技術と融合することでクローディンを標的とした創薬シーズの開発が進展していくものと期待される．[23]

京都大学・月田グループによるオクルディンおよびクローディンの発見に端を発した上皮細胞生物学の土壌に育まれ，TJを標的とした創薬研究は「吸収促進剤」から「経粘膜・経皮投与，がん治療，粘膜ワクチン開発，C型肝炎治療，脳内薬物送達，炎症性疾患治療」へとらせん的発展を遂げつつある．今年に入り，クローディンが物質輸送系において細胞内経路と細胞間隙経路のカップリングに関与していること，免疫細胞の成熟化にクローディンが関与していることなども報告されており，TJを標的とした創薬研究の発展はとどまる気配を見せていない．[24,25] 今後10年間のらせん的発展の行方を見据え，上皮細胞生物学の土壌に育まれた我が国発の創薬に向けた準備をしておくことは重要かもしれない．

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6. Claudin modulatorを用いたペプチド・タンパク性医薬品の経粘膜吸収促進

小高美樹・近藤昌夫・八木清仁

要旨

ペプチドやタンパク質などのバイオ医薬は消化酵素による分解を受けやすいうえに生体膜透過性に乏しく，侵襲性の注射による投与を余儀なくされているものが多く，患者のQOL向上に資する非侵襲性投与法の開発が創薬研究における重要課題の1つとなっている。粘膜上皮細胞層は生体外異物などの非特異的な生体内侵入に対する防御網として機能し，生体の恒常性維持に深く関わっていることから，バイオ医薬の粘膜吸収促進に際しては，この粘膜バリアを安全かつ特異的に制御する技術の開発が必要となる。本稿では，粘膜バリアの分子基盤であるclaudinを標的としたバイオ医薬の粘膜吸収改善の現状および今後の課題について概説する。

Key Words

claudin, claudin modulator, tight junction, *Clostridium perfringens enterotoxin*, 出芽バキュロウイルス, 細胞間隙経路, 粘膜吸収, C-CPE

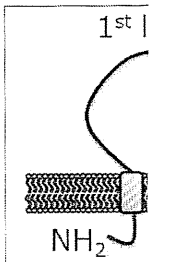
❖はじめに

周知のように，昨今次世代医薬として台頭目覚ましいペプチド・タンパク質は，消化酵素による分解を受けやすいうえに生体膜透過性に乏しく，臨床応用に際しては侵襲性の注射による投与を余儀なくされているものが多いのが現状である。患者の生活の質（QOL）および高齢社会を考慮すると，経鼻・経肺・経口投与が理想的な投与方法であるものの，元来粘膜面は生体内外を隔てるバリアとして機能しており，単に粘膜面にペプチド・タンパク質を投与しただけでは吸収性は著しく低く，粘膜バリア制御がバイオ医薬の非侵襲性投与に向けた基本戦略の1つとなっている。

さて，上皮細胞層は生体内外・組織内外を隔てる境界として機能しており，隣接する細胞の間隙に存在するtight junction（TJ）が細胞間隙における物質の漏れを抑制している[1]。当初，TJは物質透過抑制機能を有すると考えられていたが，選択的な物質透過にも関与することが示され，TJが組織固有の内部環境維持に積極的に関与していることが明らかになりつつある。このことは，TJバリアを自由自在に制御することができれば，細胞間隙経路を介した組織特異性・透過物質特異性を兼ね備えた薬物送達法開発が可能になることを示唆している。

1970年代には，TJが細胞膜に埋もれたストランド様の形態を有していることが見出されていたものの，TJの脂質ミセル説が提唱されるなどTJ分子基盤の同定は立ち遅れており，TJバリアの

図❶ Claudin



・1998年，
・分子量～2
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・TJのバリ
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本稿では，
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Ⅰ．CLs

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もTJシール
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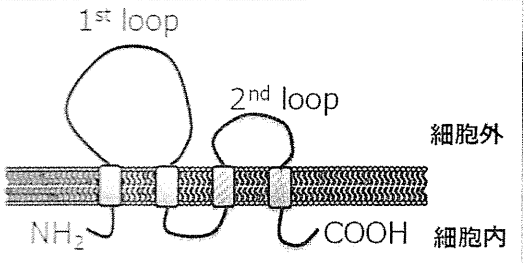
図❶ Claudin



- 1998年，京大古瀬らにより発見[5]
- 分子量～23kDa
- 4回膜貫通タンパク質
- TJのバリア機能の本体
- 24種類の分子が存在
- 発現・バリア機能が組織特異的[4]

分子基盤に立脚した薬物送達研究は遅々として進展していなかった[2,3]。1998年に京大月田グループにより4回膜貫通タンパク質claudin（CL）が同定され，1999年以降CLがTJシール機能の本体であることを示唆する知見が集積し，CLを標的とした薬物送達の概念が提唱されている[4,5]。

本稿では，CLの発見に端を発した粘膜吸収改善法の可能性および実用化に向けた課題について概説する。

Ⅰ．CLs

1993年にTJ構成タンパク質として4回膜貫通タンパク質occludinが同定され，TJシールがタンパク質によって構成されている可能性が初めて提唱された。しかしながら，occludinを欠損させてもTJシールが形成されており，引き続きTJシール機能を担う分子の同定が進められた[6]。1998年に新たなTJ構成タンパク質としてFuruseらによりCLが見出され，現在までに少なくとも24種類の分子が見出されている（図❶）[4,5]。CLの発現およびバリア機能には組織特異性が認められており，CL-1欠損マウスでは皮膚の重層上皮バリアにおける物質透過性，CL-5欠損マウスでは血液脳関門における物質透過性が亢進している[7,8]。物質透過性に分子量依存性が認められること，CLsは細胞間隙においてイオン透過経路としても機能していることから，CLバリアを制御することで組織特異性・透過物質特異性を兼ね備えた新たな薬物送達法開発の可能性が示唆される[4]。

Ⅱ．CL modulator

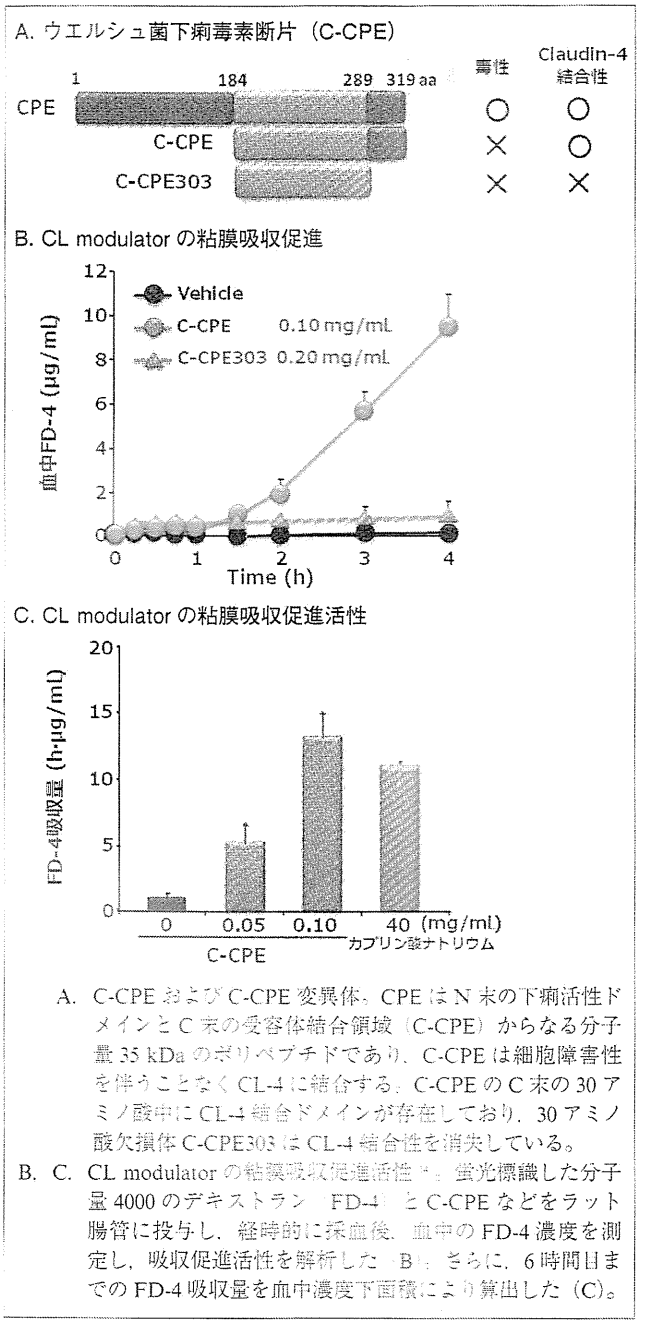
Clostridium perfringens enterotoxin（CPE）[用語1]はヒトで食中毒を引き起こすことから，長年にわたり毒素学領域において研究が進められており，CPEのN末領域が下痢活性に関与すること，C末領域が受容体結合に関与することが知られている（図❷A）[9-11]。1997年にCPEの受容体が同定され，1999年にこの受容体がCL-4と同一であることが見出された[12,13]。Sonodaらは，CPEの受容体結合領域であるC-CPE[用語2]を上皮細胞に添加することでCL-4レベルが低下し上皮細胞バリア能が減少すること，本CPE断片を除去するとCL-4レベル・上皮細胞バリア能が回復することを見出し，CL modulatorを利用した薬物送達の可能性を提唱している[12]。

著者らのグループでは，C-CPEが細胞障害性を伴うことなく上皮細胞バリア機能を低下させること，C-CPEがポリペプチドであること（C-CPEをプロトタイプとして用い遺伝子工学的手法を用いた新規CL modulator創製が可能）に着目し，C-CPEをCL modulatorのモデル分子として利用し，CLを標的とした粘膜吸収促進の可否ついての検証を試みた。

Ⅲ．CL modulatorを利用した粘膜吸収促進

分子量4000のデキストラン（FD-4）をモデル薬物として用いてラット空腸におけるC-CPEの粘膜吸収促進作用を解析したところ，C-CPEを添加することで時間・濃度依存的に血中のFD-4濃度が上昇していたこと，C-CPEのCL-4結合ドメインを欠損させることで粘膜吸収促進活性が消失していたこと，C-CPE処理に伴う粘膜傷害作用が観察されないことから，C-CPEはCL-4との相互作用を介して粘膜吸収促進活性を発揮するものと推察された（図❷B，未発表データ）。臨

図❷　CL modulator の腸管粘膜吸収促進活性



A. C-CPE および C-CPE 変異体．CPE は N 末の下痢活性ドメインと C 末の受容体結合領域（C-CPE）からなる分子量 35 kDa のポリペプチドであり，C-CPE は細胞障害性を伴うことなく CL-4 に結合する．C-CPE の C 末の 30 アミノ酸中に CL-4 結合ドメインが存在しており，30 アミノ酸欠損体 C-CPE303 は CL-4 結合性を消失している。

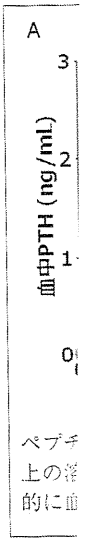
B. C. CL modulator の粘膜吸収促進活性．蛍光標識した分子量 4000 のデキストラン（FD-4）と C-CPE などをラット腸管に投与し，経時的に採血後，血中の FD-4 濃度を測定し，吸収促進活性を解析した（B）．さらに，6 時間目までの FD-4 吸収量を血中濃度下面積により算出した（C）。

床応用されている吸収促進剤であるカプリン酸ナトリウムと粘膜吸収促進活性を比較したところ，カプリン酸ナトリウム 40 mg/mL と同程度の吸収促進活性を C-CPE はわずか 0.1 mg/mL で有していた（図❷ C）。さらに，C-CPE は分子量 10000 までのデキストランについて顕著な粘膜吸収促進作用を有しており，CL modulator を利用したペプチド・タンパク質医薬の粘膜吸収促進の可能性が示唆された[14]。

次に，骨粗鬆症治療において注射剤として使用されている副甲状腺ホルモン（PTH）の粘膜吸収促進活性を検討したところ，C-CPE と PTH の共投与では経鼻粘膜吸収促進効果は観察されたものの，腸管および経肺吸収促進活性は全く観察されなかった（図❸ A，未発表データ）。C-CPE を前処理することで PTH がいずれの粘膜面でも吸収されていたことから，CL modulator はペプチド医薬の粘膜吸収促進技術として応用できるものと推察された。そこで C-CPE の立体構造情報を基に C-CPE の物性改善を試み，C-CPE の N 末を一部欠損させることで C-CPE と同程度の CL-4 結合性を保持しつつ溶解性が 10 倍以上改善された C-CPE 変異体の創製に成功した[15]（未発表データ）。新規 CL modulator と PTH を共投与することで腸管・肺胞粘膜からの吸収促進が認められ，CL を標的としたペプチド医薬の粘膜吸収促進の proof of concept を確立した（図❸ B, C）。また最近，CL modulator により抗体の粘膜吸収も改善できることを見出している。

CL は TJ においてホモおよびヘテロストランドを形成しており，24 種類存在するメンバーの多種多様な組み合わせにより様々な生体バリアを構築している。例えば，CL-1 や CL-4 は粘膜バリア，CL-1 は皮膚バリアを担っており，CL modulator の実用化に際しては投与部位に応じた CL modulator の最適化が重要となってくる。現在のところ，CL modulator としては唯一 C-CPE が CL-4 modulator として知られているのみであ

図❸　C



る。そこで
た新規 CL r
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なアミノ酸
ーを作製し
製系の構築
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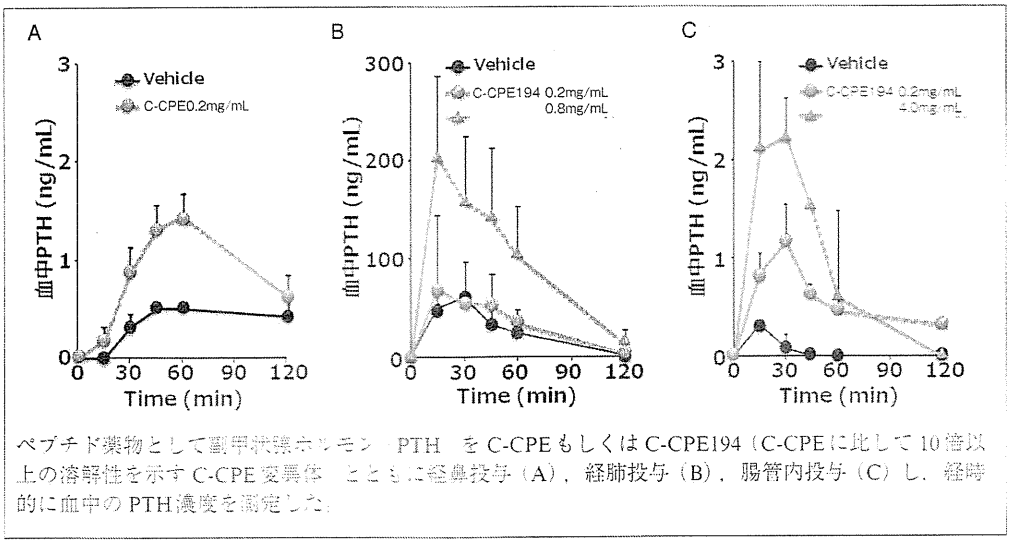
図❸ CL modulator によるペプチドの粘膜吸収促進活性（文献15より）



ペプチド薬物として副甲状腺ホルモン PTH をC-CPEもしくはC-CPE194（C-CPEに比して10倍以上の溶解性を示すC-CPE変異体）とともに経鼻投与（A），経肺投与（B），腸管内投与（C）し，経時的に血中のPTH濃度を測定した。

る。そこで，C-CPE をプロトタイプとして用いた新規 CL modulator 創製を図るために C-CPE の機能ドメインを同定し，機能ドメインをランダムなアミノ酸に置換した C-CPE 変異体ライブラリーを作製した。CL は疎水性が高くタンパク質精製系の構築は著しく立ち遅れており，CL 発現細胞などを用いたスクリーニングを試みたものの，非特異的な結合が多く新規 CL modulator の創製には成功しなかった。東大先端研の浜窪隆雄博士は出芽バキュロウイルス[用語3]（BV）の膜上に膜タンパク質がインタクトな状態で高密度提示されることを見出し，BV を利用した膜タンパク質発現系を構築している。そこで CL-4 提示 BV を作製し，C-CPE を用いた結合性解析およびスクリーニング条件の解析を試みたところ，CL 提示 BV が CL modulator スクリーニングシステムとして機能していた。現在までに，CL 提示 BV を用いて C-CPE 変異体ライブラリをスクリーニングし，多様な CL 結合活性を有する C-CPE 変異体の創製に成功している[16]（未発表データ）。今後は，本システムを有効活用することで，CL modulator のライブラリー化を図り，投与部位に応じた CL modulator の最適化を試みる予定である。

表❶ 代表的な粘膜吸収促進剤

吸収促進剤	研究開発された年代
EDTA	1980
オレイン酸	1980～
NO供与剤	1998
カプリン酸ナトリウム	1980～

表❷ 上皮細胞バリアの生物学の進展

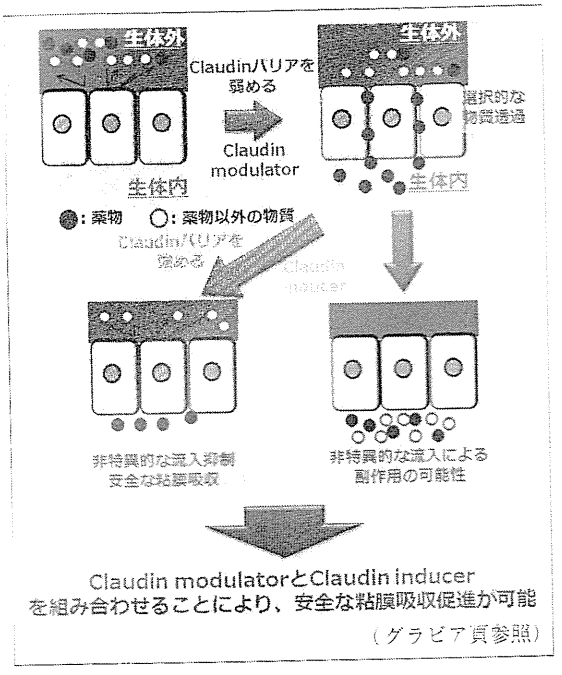
年代	上皮細胞バリアに関する発見
1993	Occludinの発見
1998	Claudinの発見
1999～	ClaudinのTJバリア機能が証明
2005	Tricellulinの発見

おわりに

周知のように，細胞間隙経路を利用した薬物送達方法は1970年代より吸収促進剤として研究が進められており，TJ シールの開口に伴う薬物以外の物質の非特異的な流入が安全な吸収促進法開発に向けた最重要課題の1つとなっている。

さて，粘膜吸収促進剤の研究年表（表❶）と上皮細胞バリアの生物学の年表（表❷）を比較してみると，吸収促進剤が研究開発されていた時代にはTJ の分子基盤が未解明であったことがわか

図❹　Claudinを標的とした粘膜吸収促進



る。このことは，TJの分子基盤に立脚したアプローチを取ることで従来の粘膜吸収促進とは異なる概念の吸収戦略の構築が可能になることを示唆している。例えば，CL-1欠損マウスでは分子量800程度の分子について皮膚透過性が亢進すること，CL-5欠損マウスでは分子量1000未満の分子の血液脳関門透過性が亢進すること，CLが細胞間隙経路を介したイオン透過経路として機能していることなどが報告されている[4,7,8]。これらの知見は，CLを制御することで細胞間隙経路を介した分子量・荷電依存的な薬物送達が可能になることを示唆しており，CL modulatorは投与部位特異性および透過物質特異性を有する安全性に優れた粘膜吸収促進法としての応用が期待される。これまでのところ，*in vivo*で吸収促進活性を有するCL modulatorとしてはC-CPEsしか報告されておらず，頻回投与での抗原性が危惧されている。目下，当研究グループをはじめとした複数のラボでC-CPEの立体構造解析，C-CPE・CL複合体の立体構造解析が進められており，近い将来，立体構造情報を基にしたchemical・低分子CL modulatorが創出されるものと期待される。

CL modulatorは可逆的にCLバリアを阻害できるものの，安全性確保に際しては薬物吸収後に可及的速やかにCLバリアを回復させることが重要となる。われわれのグループでは，レポーター遺伝子を利用した迅速かつ簡便なCL inducer探索系を構築し，本システムを用いてすでに複数のCL-4 inducerの取得に成功しており，CL modulatorとCL inducerを融合することで生体バリアを自在に制御する方法論の確立を進めているところである（図❹）。

以上，1998年のCLの発見に端を発した上皮細胞バリアの生物学の進展には目覚ましいものがあり，新たな粘膜吸収促進法開発に向けた萌芽が生まれつつある。今後は，CLバリア制御に伴う副作用発現の可否の検証や低分子型CL modulatorの創製などの課題を1つずつクリアすることで，バイオ医薬を本邦独自の創剤力で非侵襲性投与可能にする技術が実用化するものと確信している。

謝辞
本稿を執筆する機会を与えていただいた京都薬科大学山本昌先生をはじめとした関係者の皆様に衷心よりお礼申し上げます。本稿で紹介したCL modulatorの研究成果は文部科学省科学研究費補助金（課題番号：21689006），文部科学省地域イノベーションクラスタープログラムのサポートにより実施されたものである。

用語解説

1. *Clostridium perfringens enterotoxin*（CPE）：ウエルシュ菌による食中毒の原因となっている35 kDaの毒素。N末側に存在する下痢活性に関与する領域とC末側に存在する受容体結合領域からなる。1997年に本毒素の受容体は同定されていたが，1999年に月田承一郎博士らのグループによってclaudin-4が本受容体と同一分子であることが見出された。
2. C-CPE：CPEの受容体結合領域であるC末側14 kDaのポリペプチド断片。1999年，月田承一郎博士らのグループにより，C-CPEがclaudin-4のシー

…機能を阻害することが見出され，claudinが上皮細胞層の密着結合におけるシール機能の本体であることが証明された．その後，ノックアウトマウスの作出などにより，claudinのシール機能を証明するデータが蓄積．

3. 三芽バキュロウイルス：膜タンパク質を機能および活性を保持した状態で発現する技術。東京大学先端科学研究センター 浜窪隆雄博士らによって開発された．バキュロウイルスは昆虫細胞に感染するウイルスであり，核内封入体と発芽ウイルスの2つの生活環をもっており，組換えバキュロウイルスを用いた昆虫細胞におけるタンパク質発現系は組換えタンパク質発現系として汎用されている．浜窪らは，発芽ウイルス（＝出芽ウイルス）の表面に組換え膜タンパク質が発現していること，ウイルス表面上の膜タンパク質が形態・機能両面においてインタクトな状態を保持していることを見出し，膜タンパク質発現技術として確立した．

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Original Paper

Pathobiology

Pathobiology 330169
DOI: 10.1159/000330169

Received: April 5, 2011
Accepted after revision: June 6, 2011

Anti-Angiotensin and Hypoglycemic Treatments Suppress Liver Metastasis of Colon Cancer Cells

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Key Words
Angiotensin · Renin · Hyperglycemia · Colon cancer · Liver metastasis

Abstract
The effect of diabetic conditions on liver metastasis was examined using CT26 mouse colon cancer cells. CT26 cells produced angiotensin (A)-I and A-II from angiotensinogen; the production was abrogated by inhibitors of renin and chymase. Renin expression and A-II production increased with an increase in the concentration of glucose in the medium. In a streptozotocin-induced BALB/c mouse diabetes model that was fed a high-calorie diet, the blood sugar level increased and was associated with an increasing size and number of CT26 liver metastases. In this diabetic mouse model, liver metastasis of CT26 cells was suppressed by anti-angiotensin treatment with a chymase inhibitor, a renin inhibitor, and an A-II receptor blocker. Moreover, concurrent hypoglycemic and anti-angiotensin treatments showed a synergistic inhibitory effect on CT26 cell liver metastasis. These results suggest that angiotensin activation ability associated with diabetic conditions enhances liver metastasis of colon cancer. Therefore, treatment with anti-angiotensin and hypoglycemic agents might be relevant for baseline management of colon cancer patients with the diabetic condition for the prevention of liver metastasis. This scheme needs to be examined in a clinical setting.

Introduction

Colorectal cancer (CRC) is the third leading cause of cancer death in Japan and the leading cause of cancer death in women. CRC mortality continues to increase as the Western dietary style is gaining popularity in Japan [1]. About 30% of CRC patients die from liver metastasis [2], and the 5-year survival rate of CRC patients with liver metastasis is less than 20% [1]. Thus, the diagnosis and treatment of liver metastasis is a pivotal problem in conquering CRC.

Type 2 diabetes is one of the most notorious outcomes of the Western dietary style. Diabetes is associated with

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increased disease risk and CRC mortality [3, 4]. The blood level of glycated hemoglobin (HbA1c) is considered a marker of CRC risk [5]. The mechanism underlying the protumoral role of diabetes is explained by the significance of plasma insulin levels [3]. An increased blood concentration of insulin and insulin-like growth factor enhances CRC risk [6].

Angiotensin-II (A-II) is known to be a protumoral factor which leads to vasoconstriction and proliferation in neoplastic tissues [7]. A-II induces the activation of protein kinase C and the induction of angiopoietin 2, vascular endothelial growth factor (VEGF), VEGF receptors, fibroblast growth factor, platelet-derived growth factor, transforming growth factor beta, epidermal growth factor, nitric oxide synthase, and metalloproteinase [7, 8]. These properties enhance colon cancer progression, particularly its metastasis.

In the present study, we aimed to elucidate the antimetastatic effect of anti-angiotensin and hypoglycemic therapies in a hyperglycemic condition using a streptozotocin (STZ)-induced mouse diabetes model.

Materials and Methods

Cell Culture and Reagents

The CT26 mouse colon cancer cell line was kindly provided by Prof. Isaiah J. Fidler (MD Anderson Cancer Center, USA) [9]. Cells were maintained in Dulbecco's modified essential medium (Sigma Chemical Co., St. Louis, Mo., USA) containing 10% fetal bovine serum (Sigma) under conditions of 5% CO_2 in air at 37°C. The glucose concentration was 200 mg/dl in the regular medium. Angiotensinogen (ATG, 40 nM; Calbiochem, Darmstadt, Germany), D-glucose (Sigma), aliskiren (ALI; renin inhibitor, 1 μM; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif., USA), chymostatin (CMS; chymase inhibitor, 50 μM; Peptide Research Institute), and captopril (CAP; ACE inhibitor, 1 mM; MP Biomedicals LLC, Solon, Ohio, USA) were purchased.

Immunoblot Analysis

Whole-cell lysates were prepared as described previously [10]. Anti-renin antibody (AnaSpec, Inc., San Jose, Calif., USA) was used as the primary antibody. An anti-tubulin antibody was used as a loading control (Oncogene Research Products, Cambridge, Mass., USA). The immune complex was visualized using an enhanced chemiluminescence Western blot detection system (Amersham, Aylesbury, UK). Whole-cell lysates were also used for ELISA.

Animal Model

BALB/c mice (male, 4 weeks old) purchased from Japan SLC, Inc. (Shizuoka, Japan) were used as a metastasis model. The mice were maintained according to the institutional guidelines approved by the Committee for Animal Experimentation of Nara Medical University. Single-cell suspensions of CRC cells (1×10^6) in Hank's balanced saline solution were injected into the mouse spleen. The mice were sacrificed to determine the number and size of metastatic foci in the liver [11].

Mice were fed a normal diet (CE-2; total calories 343.1, NFE 50%, crude fat 4.8%, mainly consisting of soybean oil; Clea Japan, Inc., Tokyo, Japan) or a high-calorie diet (HCD; QuickFat, total calories 405.5, NFE 46.5%, crude fat 13.6%, mainly consisting of beef tallow; Clea Japan) with or without 10% glucose consumption (Otsuka Pharmaceutical Co., Tokushima, Japan). Mice were treated with STZ (200 mg/kg of body weight, i.p., once; Wako Pure Chemical Industries, Ltd., Osaka, Japan) for a diabetes model, gliclazide (GCZ; 3.7 mg/kg of body weight, p.o., in the evening, once a day; Wako), porcine insulin (INS; 4 units/kg of body weight, s.c. in the evening, once a day; MP Biomedicals), ALI (25 mg/kg of body weight, s.c., in the evening, once a day), losartan (LOS; 2 mg/kg of body weight, intragastric administration, in the evening, once a day; Toronto Research Chemicals, Inc., Toronto, Ont., Canada), or CMS (10 mg/kg body weight, i.p., in the evening, once a day).

Mouse blood sugar and A-II in serum or cultured medium were measured using a Medisafe Reader (Terumo Co., Tokyo, Japan) and A-II ELISA kits (Phoenix Pharmaceuticals, Inc., Belmont, Calif., USA), respectively. Renin and chymase protein levels in whole-cell lysates were examined using ELISA kits (Uscn Life Science, Inc., Wuhan, China; and DRG International Inc. East Mountainside, N.J., USA, respectively). ELISA was performed according to the provider's instructions.

Statistical Analysis

Statistical analyses of experimental data were performed using the Mann-Whitney U test and the Kruskal-Wallis test with Dunn's multiple comparisons test (nonparametric ANOVA). The survival rates of mice were examined using the Kaplan-Meier method. A two-sided $p < 0.05$ was considered statistically significant.

Results

Angiotensin Activation by CT26 Cells

We examined the angiotensin activation ability of CT26 cells (fig. 1a). CT26 cells produced A-I and A-II from ATG. CT26 cells were treated with inhibitors of angiotensin activation-related enzymes. Treatment with chymase inhibitor abrogated A-II production and increased A-I levels. Renin inhibitor abrogated the production of A-I and A-II. In contrast, ACE inhibitor did not affect the production of A-I or A-II. These results suggest that CT26 cells activate angiotensin via renin and chymase. The A-II level depended on the glucose concentration of the culture medium (fig. 1b). When CT26 cells were cultured with different concentrations of glucose, the renin protein levels were increased in a dose-dependent manner. In contrast, chymase production was not altered by the glucose concentration (fig. 1c).

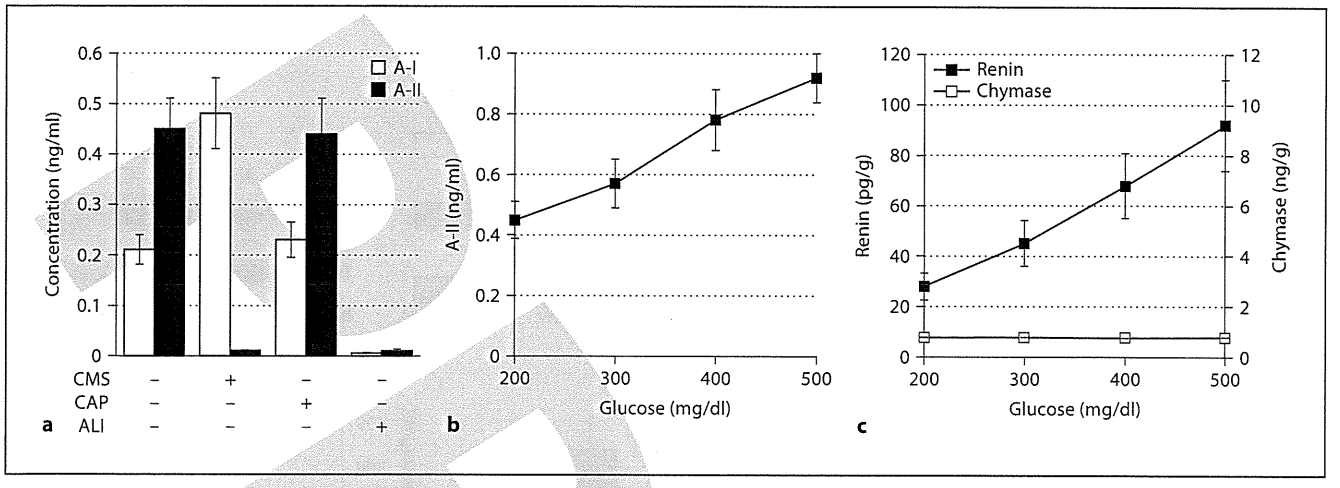

Fig. 1. Angiotensin activation in CT26 cells. **a** The A-II concentration in the culture medium was examined by ELISA. CT26 cells were treated with ATG (40 nM) and CMS (50 μM) or CAP (1 mM) or ALI (1 μM) for 12 h. **b** A-II concentration in the culture medium. CT26 cells were cultured in a medium containing various concentrations of glucose. **c** Renin and chymase protein levels in the CT26 cells were examined by ELISA. Error bars represent the SD.

Effect of Hyperglycemia on Liver Metastasis of CT26 Cells

To examine the effect of hyperglycemia on liver metastasis of CT26 cells, nude mice fed an HCD and/or given STZ injection were used (fig. 2). The body weights of the mice in each group did not significantly differ; however, the blood sugar level was higher in the HCD, STZ, and combined HCD/STZ groups (groups B, C, and D) than in the control group (group A) ($p < 0.0001$) (fig. 2c, d). Renin expression levels were associated with blood sugar levels (fig. 2b). The size and number of metastatic foci were higher in the STZ-treated groups (groups B and D) than in the control group (group A) (fig. 2e, f). These results suggest that a high blood sugar level enhances liver metastasis by increasing renin expression.

Inhibitory Effect of Anti-Angiotensin Agents on Liver Metastasis

To evaluate the anti-metastatic effect of anti-angiotensin agents (fig. 3), a liver metastasis model using mice fed an HCD and treated with STZ injection was modified by the administration of chymase-I (group F), renin-I (group G), and A-II type 1 receptor blocker (ARB; group H) (fig. 3a). Fewer metastatic foci were found in the groups treated with anti-angiotensin agents (groups F, G, and H) compared to the untreated group (group E) ($p < 0.001$) (fig. 3c, d). Moreover, the overall survival in the treated groups was improved compared to that in the untreated group (fig. 3b). Thus, anti-angiotensin treatment is effective in suppressing colon cancer liver metastasis.

Inhibitory Effect of Hypoglycemic Agents on Liver Metastasis

Finally, we examined the effect of concurrent treatment with anti-angiotensin and hypoglycemic agents on the liver metastasis of CT26 cells (fig. 4). Insulin and GCZ were administered with or without renin-I in the liver metastasis model using mice fed an HCD and treated with STZ injection (fig. 4a). Treatment with insulin and GCZ resulted in lower blood sugar levels compared to those in the untreated mice (fig. 4b). The mice treated with hypoglycemic agents (groups J and K) showed a decrease in the number of metastatic foci and improved survival compared to the untreated group (group I) (fig. 4d, e). Concurrent treatment with anti-angiotensin and hypoglycemic agents (groups L and M) resulted in a lower serum A-II concentration, a smaller number of metastatic foci, and longer survival compared to the untreated mice (group I) or the mice treated with hypoglycemic agents alone (groups J and K) (fig. ■■■c–e). The mice treated with the combination showed suppression of liver metastasis and improved survival which was indistinguishable from that of the control mice (group N) (fig. 4d, e).

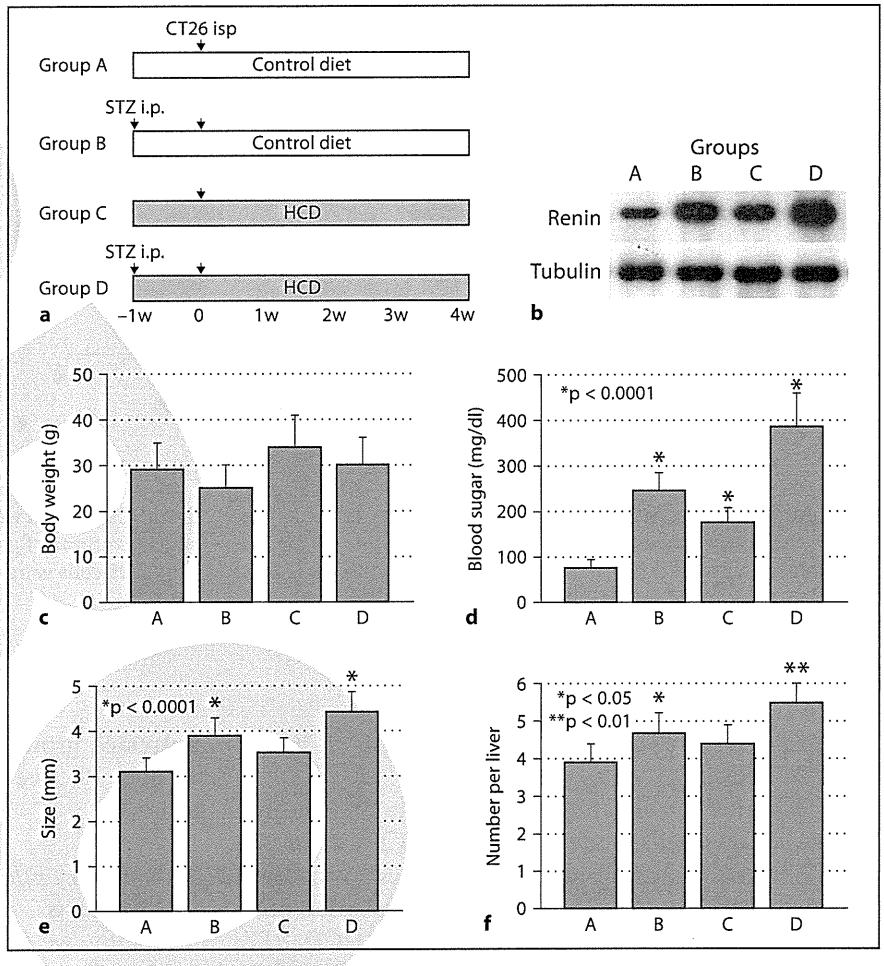

Fig. 2. Effect of STZ-induced diabetic conditions on liver metastasis of CT26 mouse colon cancer cells. **a** Protocol of a liver metastasis model using the STZ-induced diabetic mouse. Mice were fed a control diet or an HCD. Each group consisted of 6 mice. **b** Renin protein expression was examined by immunoblotting in groups A, B, C, and D. Tubulin was examined as a loading control. **c–f** Body weight, blood sugar, and size and number of liver metastatic foci in each group. Error bars represent the SD.

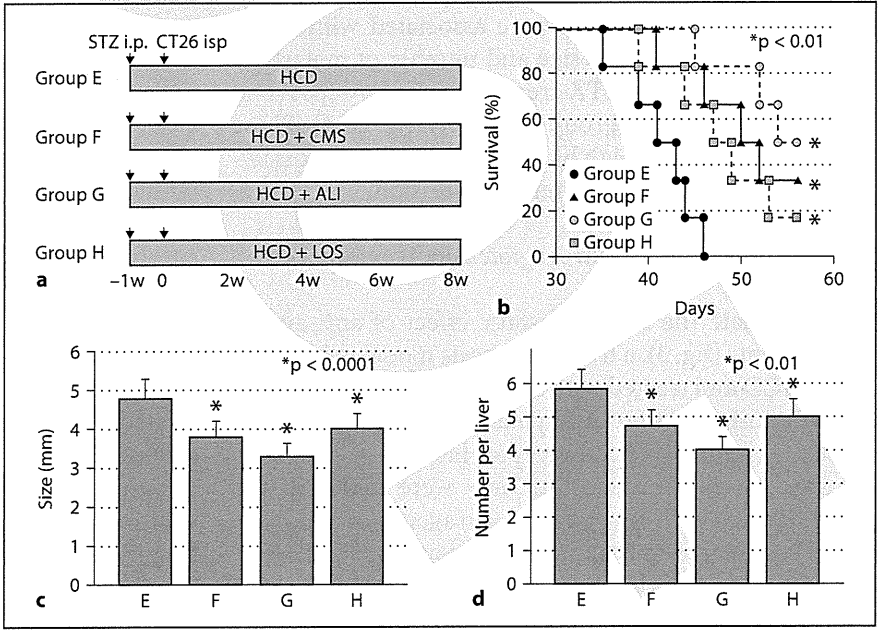

Fig. 3. Effect of anti-angiotensin agents on liver metastasis of CT26 mouse colon cancer cells. **a** Protocol of a liver metastasis model using STZ-induced diabetic mice. Mice were treated with chymase inhibitor (chymase-I, CMS), ALI, and losartan (LOS). Each group consisted of 6 mice. **b** The survival of mice in each group was calculated and compared using the Kaplan-Meier method. Statistical significance was compared between group E and all other groups. **c**, **d** Size and number of liver metastatic foci in each group. Error bars represent the SD.

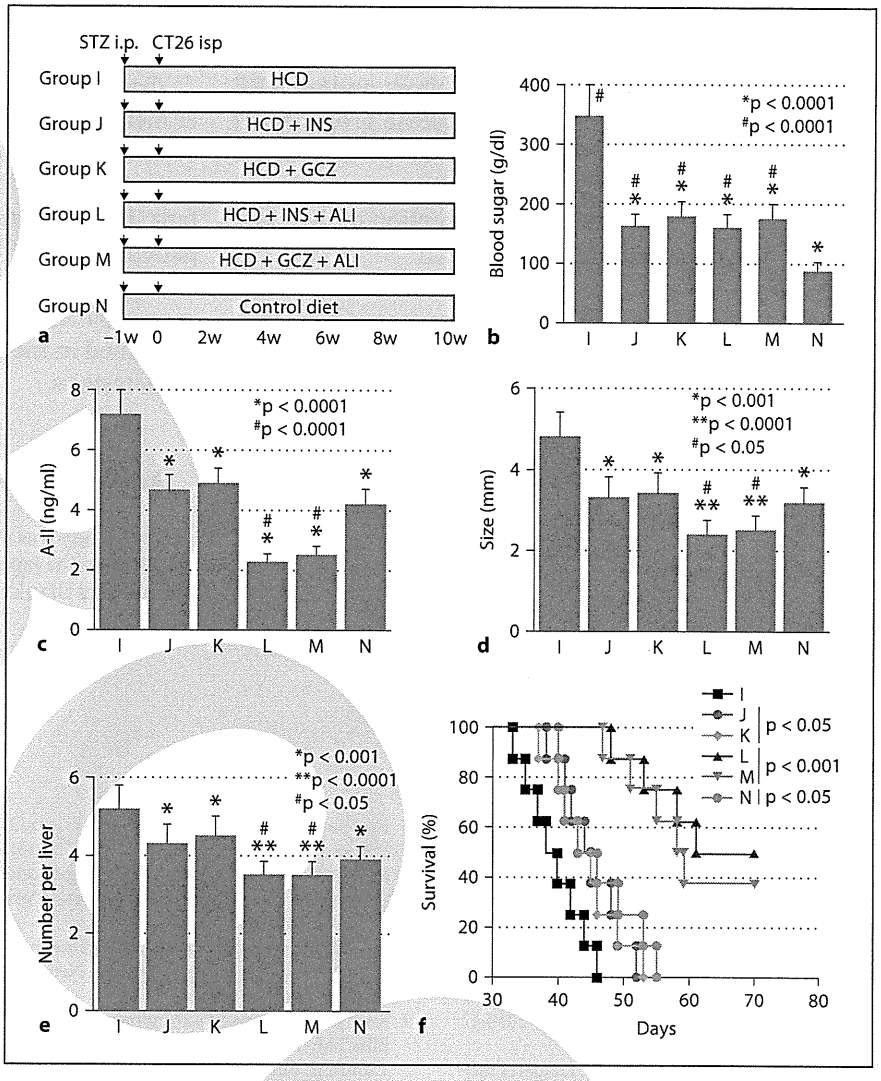

Fig. 4. Effect of concurrent treatment with an anti-angiotensin agent and a hypoglycemic agent on liver metastasis of CT26 mouse colon cancer cells. **a** Protocol of a liver metastasis model using STZ-induced diabetic mice. Mice were treated with a combination of ALI and INS or GCZ. There were 8 mice in each group. **b–e** Blood sugar, serum A-II, and size and number of liver metastatic foci in each group. Error bars represent the SD. **f** The survival of mice in each group was calculated and compared using the Kaplan-Meier method. Statistical significance was compared between group I and all other groups.

Discussion

CT26 CRC cells possess an angiotensin activation mechanism provided by expression of renin and chymase. In fact, CT26 cells express renin in association with the glucose concentration in a dose-dependent manner. The angiotensin activation ability of CT26 cells was confirmed by A-II production which was inhibited by both a renin inhibitor and a chymase inhibitor. Thus, the renin-chymase system is thought to be employed to activate ATG in CT26 cells. Renin expression was induced by the glucose in the culture medium. A high glucose level increases renin expression and the subsequent A-II production in the cardiac fibroblasts [12]. We also found that renin expression was increased by the hyperglycemic status in the STZ-induced diabetic mice. Moreover, hyperglycemia also enhanced liver metastasis in the STZ-induced diabetic mice. These findings suggest that hyperglycemia in diabetic animals enhances liver metastasis via angiotensin activation.

Based on these results, we examined the effects of anti-angiotensin treatment and/or hypoglycemic treatment on liver metastasis of CT26 cells. Inhibitors of the renin-angiotensin system are widely used to treat hypertension. In the present study, some anti-angiotensin agents, inhibitors of renin and chymase, and ARB suppressed liver metastasis of CT26 cells. Moreover, hypoglycemic treatment by GCZ and insulin showed improvement of liver

metastasis. Combined treatment with anti-angiotensin and hypoglycemic agents showed a synergistic inhibitory effect on liver metastasis. ACE inhibitors and/or ARB have been reported to improve the disease prognosis or reduce progression in pancreatic and urogenital cancer [13, 14]. Our results suggest that anti-angiotensin system therapy should also be tested for prevention of liver metastasis in colon cancer.

In the present study, we used an STZ-induced mouse diabetes model. Since STZ damages pancreatic beta cells, this is not really the most appropriate model to study type II diabetes [15]. The STZ dosage used in this study results in partial damage to the beta cells which maintain insulin production at levels lower than those in normal mice (data not shown). We fed the mice an HCD which results in a metabolism resembling that observed in type II diabetes [16]. Thus, the STZ-induced diabetes model combined with an HCD is a suitable simulation of type II diabetes [15]. To confirm the effect of diabetic conditions on liver metastasis, further experiments using spontaneous or genetically engineered diabetes model rodents are required.

Considering that hyperglycemia is associated with liver metastasis of colon cancer via renin upregulation, diabetic status is thought to be a risk factor for liver metastasis. Control of blood sugar could, therefore, be important in preventing liver metastasis in colon cancer patients. The effect of anti-angiotensin treatment and blood sugar control for baseline management of colon cancer patients with the diabetic condition needs to be examined in the clinical setting for the prevention of liver metastasis.

Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, Japan. The authors thank Dr. Mamoru Isobe for supporting the statistical analyses.

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Curr. Res. in Cancer 5, 2011 (1-10)

HMGB1 induces drug resistance in cancer cells

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Abstract

HMGB1 is a non-histone chromosomal protein, a secretory protein binding to the receptor for advanced glycation end products in cancer cells and monocyte-lineage immune cells. HMGB1 enhances proliferation, motility, invasion, and survival of cancer cells. HMGB1 associated with DNA repair of anti-cancer drug-induced DNA damage. Importantly, HMGB1 is released from necrotic cancer cells and induces re-growth of the remnant cancer cells. In contrast, HMGB1 induces apoptosis in monocyte-lineage immune cells and inhibits tumor-infiltrating macrophages and dendritic cells, lymph node sinus macrophages, liver Kupffer cells to attenuate anti-cancer immune responses and anti-metastatic organ defense.

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Introduction

High motility group box (HMGB)-1 is a multifunctional protein possessing diverse biological activities in normal cells. The roles of HMGB1 in cancer are also diverse and can be divided into 2 categories: its direct effect on cancer cells, and its effect on host immunity. HMGB1 provides pro-tumoral and anti-immune effects in the cells expressing the receptor for advanced end glycation products (RAGE). Both cancer cells and monocyte-lineage cells express RAGE; however, the effect is completely different between the two cells. Essentially, HMGB1 accelerates the metastasis of cancer cells. In this review article, we describe the roles of HMGB1 in cancer and immunity, and anti-cancer drug and tumor re-growth after anti-cancer treatment. The significant roles of HMGB1 in cancer suggest that HMGB1 is an excellent molecular target for cancer treatment, especially anti-metastatic therapeutics. These roles of HMGB1 after all provide resistance to anti-cancer drugs.

HMGB1

The high mobility group box 1 (HMGB1) protein is one of several non-histone chromosomal proteins found in eukaryotic cells (1-3). HMGB1 is isolated as a cytosolic 30-kDa protein from fetal brain tissue (4, 5) and is associated with neurite outgrowth (2, 3). As a nuclear protein, HMGB1 binds to DNA participating in multiple processes such as transcription, replication, recombination, DNA repair, and genomic stability (6).

In the cytoplasm, HMGB1 is associated with cell motility as observed in the outgrowing neuritis. At the leading edge of the motile cell, HMGB1 accelerates formation of filopodia as well as actin-polymer formation (2). The mechanism of HMGB1-dependent cell migration in cancer cells is considered to be similar to that of outgrowing neurites. HMGB1 is expressed in immature cells and malignant cells at high levels and it plays a major role in controlling cell migration activity (7). The DNA-binding capacity of HMGB1 provides a role as a DNA chaperon. In the innate immune system, HMGB1 is the effective DNA sensor presenting the DNA to toll-like receptor (TLR) (8). Recently, endogenous HMGB1 is revealed to activate an autophagy signal, which promotes cell survival (9).

HMGB1 is secreted from activated monocytes, macrophages, and NK cells, and acts extracellularly as a proinflammatory cytokine. HMGB1 expression and secretion is upregulated in response to stimulation of cells by proinflammatory cytokines, endotoxin, and oxidative stresses in macrophages (10-13). Cancer cells also overexpress and secrete HMGB1 by stimulation of growth factors, cytokines, and

cellular stresses involving advanced glycation end products (AGE) and deoxycholic acid (14-17). Secreted HMGB1 activates RAGE as a ligand to induce cell growth, motility, invasion, and angiogenesis as will be described below.

HMGB1 receptor

The HMGB1 receptor, RAGE is purified from bovine lung endothelial extract as receptor of AGE (18). RAGE, which is a member cell surface receptors belonging to the immunoglobulin superfamily (19-22). RAGE is closely associated with cell growth, cell invasion through mitogen-activated protein (MAP) kinase activation, and matrix metalloproteinase (MMP)-2 and -9 expression in glioma cells (22). RAGE upregulation is found in colon and oral carcinogenesis in rodents (16, 23).

The co-expression of HMGB1 and RAGE is pivotal for accelerating tumor metastasis and poor prognosis in glioma, gastric, colorectal, and prostate cancer (15, 22, 24-26). Gastric and colon cancer cells show concurrent expression of HMGB1 and RAGE, which is closely associated with the autocrine/paracrine regulation of cell motility and invasion of cancer cells (15, 24-26). Metastatic prostate cancer cases show HMGB1 induction in prostatic stromal cells. Concurrence of RAGE expression in tumor cells and HMGB1 expression in stromal cells accelerate cancer metastability (15).

In contrast, high-level expressions of RAGE and HMGB1 are found in normal lung tissue and non-small cell lung cancer, which, in contrast with other cancer, is associated with tissue differentiation and good prognosis (27, 28). RAGE is also associated with myogenic differentiation of myoblasts and rhabdomyosarcoma, which is associated with reduction of malignant phenotypes of the disease (29, 30).

HMGB1 secretion

HMGB1 is released by both active and passive processes. HMGB1 is actively transported from the nucleus to the cytoplasm following detachment from loosened chromosomes by histone acetylation (17). And recent studies have shown that, it shuttles between the nucleus to the cytoplasm through hyperacetylation and phosphorylation in macrophages, and is monomethylated at Lys42 in neutrophils. HMGB1 is released from necrotic cells by passive diffusion (31). However, HMGB1 is not released from tightly packed nuclei of apoptotic cells and triggers inflammation..

HMGB1 intracellular signals

The interaction of HMGB1 with RAGE also activates the intracellular signaling pathway of MAP kinase. Consequently, RAGE activates GTPases, Ras, Cdc42, Rac, Rho, and MMP-2/-9 (3, 22). RAGE expression is associated with cell invasion

(24, 26) and it is suggested that type IV collagenase activation may be one mechanism for enhancement of the invasive capacity of cancer cells. RAGE activation induces cell growth through MAP kinase signaling (22). RAGE activation is also associated with induction of inducible nitric oxide synthase (iNOS), nuclear factor (NF) kB activation, and Bcl-2 production (32). NFkB activation is associated with HMGB1-dependent chemotaxis (33).

HMGB1 and angiogenesis

Intracellular signaling pathways of RAGE induce vascular endothelial cell growth factor (VEGF) expression and activate NF-kB in vascular endothelial cells (34). Activated RAGE induces VEGF expression transcriptionally through activation of NFkB, AP-1, and hypoxia-inducible factor (HIF)-1α (34, 35), which is also associated with the complications in diabetes, such as diabetic retinopathy (36). There is a difference in of VEGF induction between AGE and HMGB1 (26). AGE-BSA has a more pronounced effect on VEGF expression than HMGB1 in colorectal cancer cell lines. In our studies, HMGB1 induced the secretion of VEGF but not that of VEGF-C in human oral squamous cell carcinoma (OSCC) cell lines (37). VEGF-C and VEGF-D are associated with lymph node metastasis (38). Differential induction of VEGF from VEGF-C through activation of RAGE by HMGB1 may explain why RAGE expression is not associated with lymphangiogenesis. Lymph node metastasis of cancer is strongly associated with lymphangiogenesis (39).

HMGB1 in anti-cancer immunity

HMGB1 is associated with a significant reduction of intratumoral macrophage infiltration in metastatic colon cancer (40). HMGB1 induces growth inhibition in rat peritoneal macrophages, U937 human monocytic cells, and human alveolar macrophages, and induces apoptotic death with phosphorylation of JNK and Rac1, and upregulation of caspase-3 and caspase-9 (41, 42). JNK is associated with apoptotic signals transmitted by Rac1/Cdc42 (29, 30, 43).

Tumor-associated macrophages also have anti-cancer effects (44). In clinical studies, colon cancer patients with high-level macrophage infiltration show less invasion and metastasis than those with low-level macrophage infiltration (45). Depletion of tumor-infiltrating macrophages is closely associated with advanced stages of human colon cancer and with metastatic ability in a mouse colon cancer model (40). Dukes B CRC cases with macrophage-cancer cell contact, whereas Dukes C cases showed no such contact. HMGB1 expression is associated with macrophage depletion in colon cancer tissues (40).

Lymph sinus macrophages and liver Kupffer cells (KCs) participate in the immune response of the organs against metastatic cancer cells. Sinus macrophages and KCs mediate the phagocytosis of cancer cells attached to the sinus wall in order to inhibit their metastasis (46, 47). In CRC cases, macrophage numbers in the regional lymph nodes are decreased in both non-metastasized and metastasized nodes in Dukes C cases, whereas macrophage numbers in Dukes B nodes are higher (48). Nodal HMGB1 concentration is higher in Dukes C nodes than that in Dukes B nodes; this is inversely correlated with macrophage numbers. Nodal HMGB1 concentration is correlated with HMGB1 concentration and lymph vessel density found in the primary tumors (48). High concentration of HMGB1 is reported in effusions from cancer patients. These data indicate that HMGB1 secreted from primary tumors is delivered to the regional lymph nodes and decreases the number of macrophages to weak the anti-metastatic defense of the lymph nodes in patients with CRCs.

In a nude mouse liver metastasis model, the cecal administration of HMGB1 decreased the number of KCs and increased the embedment of colon cancer cells in a dose-dependent manner (49)]. HMGB1 is secreted from primary tumors of colon cancer and delivered to the liver through portal blood flow. Following this, HMGB1 inhibits KCs to accelerate liver metastasis of colon cancer. In clinical studies, higher HMGB1 concentrations are found in the primary tumors and metastatic foci, and fewer KCs are found in Dukes D cases than in Dukes C cases. The portal blood HMGB1 concentrations are higher in Dukes D cases than in Dukes C cases, and we have shown that the concentration of HMGB1 in the portal blood is strongly correlated with the concentration of HMGB1 in the primary tumors (49). As a result, HMGB1 affects the host immunity in the metastasis-target organs in a humoral manner. Large amounts of secreted HMGB1 can affect remote organs such as the target organs of metastases from CRCs.

Dendritic cells (DCs) play a crucial role in host immune response to various extrinsic microorganisms and also to cancer cells (50). Dendritic cell densities in primary tumors and metastatic tumors are suppressed (51). Indeed, nodal metastasis-positive colon cancer cases show higher HMGB1 concentrations in lymph nodes and primary tumor tissues, and fewer dendritic cell numbers (42). HMGB1 produced by colon cancer cells resulted in a suppression of nodal dendritic cells to attenuate host anti-cancer immunity. HMGB1 results in activation of monocytes and dendritic cells; however, high concentrations of HMGB1 result in a death signal to dendritic cells, as found on macrophages (42). Mouse

peritoneal macrophage-derived dendritic cells (PMDDCs) treated with HMGB1 show a decrease in cell number in a dose-dependent manner. HMGB1-treated PMDDCs show apoptosis and increased levels of phosphorylated JNK, and intraperitoneal administration of HMGB1 decreased splenic dendritic cells in C57BL mice (42).

HMGB1 may provide cancer cells with the advantages of cancer progression and suppression of host immunity; therefore, further examination of the role of HMGB1-induced macrophage apoptosis in cancer may provide novel therapeutic targets against these diseases.

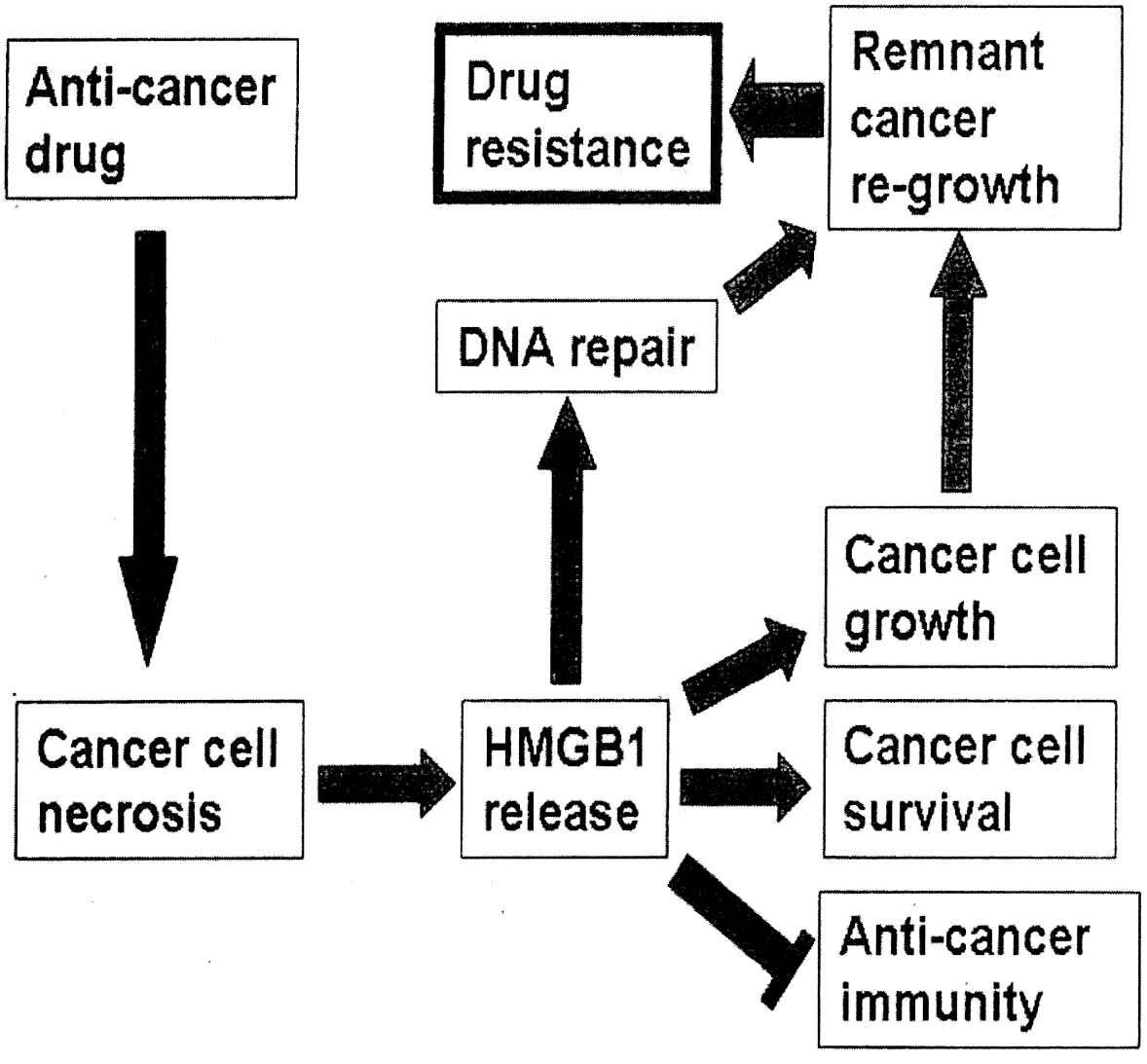

Figure 1. HMGB1 provides drug resistance in cancer cells by accelerating by increase of DNA repair, enhancement of survival and growth of cancer cells, and suppression of anti-cancer immunity.

HMGB1 and anti-cancer drugs

They bind with high affinity to specific structural distortions in the double helix such as synthetic four way junctions and adducts that are formed in DNA modified by the anti-tumor drug cisplatinum and UV light (52, 53). DNA bound HMGB1 plays a role in DNA repair providing drug resistance to platinum derivatives in cancer cells (54).

HMGB1 is passively secreted from necrotic cells. We confirm that necrosis inducers, such as doxorubicin (DXR) increase HMGB1 concentration in the cultured medium. In contrast, apoptosis inducers, such as trichostatin A (TSA) do not increase HMGB1 in the cultured medium. In a mouse tumor model of bilateral scapular subcutaneous tumors, induction of necrosis at one tumor by DXR enhances growth of the contralateral tumor. In contrast, induction of apoptosis at one tumor by TSA does not affect growth of the contralateral tumor. Moreover, in mouse liver and lung metastasis models with one subcutaneous tumor, induction of necrosis at the subcutaneous tumor by DXR increases metastasis to the liver and lung. The enhancement of metastasis is abrogated by administration of anti-HMGB1 antibody. These findings suggest that HMGB1 enhances growth of the remnant cancer cells to increase the tumor relapse and metastasis. The pro-apoptotic but not pro-necrotic anti-cancer drugs are needed to avoid HMGB1-induces cancer relapse and metastasis.

Conclusion

Resistance to anti-cancer drugs is provided primarily by abrogation of the pharmacological mechanism of the drugs. Multiple drug resistance (MDR) gene product, P glycoprotein reduced intracellular drug concentration by pump out the drug. Drug resistance is provided secondarily provided by enhancement of tumor survival and reduction of anti-cancer immunity. HMGB1 accelerates drug resistance of cancer cells by increase of DNA repair, suppression of anti-cancer immunity and enhancement of survival and growth of cancer cells (Figure 1). In this context, HMGB1 is a pivotal anti-cancer drug resistant factor. To increase the efficacy of anti-cancer treatment, HMGB1 is a relevant target.

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