労災疾病臨床研究事業費補助金

悪性胸膜中皮腫のヒト化 CD26 抗体療法確立のための予後・治療効果予測バイオマーカーの開発

平成 30 年度　総括・分担研究報告書

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Ⅰ．総括研究報告

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Ⅰ．　総括研究報告

労災疾病臨床研究事業費補助金
総括研究報告書
悪性胸膜中皮腫のヒト化 CD26 抗体療法確立のための
予後・治療効果予測バイオマーカーの開発

研究代表者　　森本　幾夫　順天堂大学大学院医学研究科
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研究要旨

悪性胸膜中皮腫はアスベストばく露によって起こる難治性悪性腫瘍であり、現時点で満足できる治療法はなく、新たな治療法の確立が望まれる。われわれは、新規治療標的分子として悪性胸膜中皮腫に発現する CD26 に着目し、ヒト化 CD26 抗体を開発しフランスにて第 I 相臨床試験を行った。安全性が確認されるとともに治療薬としての有効性を示唆する結果も得られ、平成 29 年 7 月から国内でも第 I/II 相臨床試験を開始した。平成 30 年 3 月に第 I 相臨床試験最終患者への投与が終了し、同年 6 月からスタートした第 II 相臨床試験も患者のリクルートが順調に進んでいる。今年度は悪性中皮腫患者と健常者の血清中サイトカイン・ケモカインの多項目解析を行い、一部の悪性中皮腫患者では IL-10, MIF, Eotaxin/CCL11, IL-1β の濃度が顕著に高いことを見出した。また、国内第 I/II 相臨床試験患者の末梢血リンパ球のフェノタイプ解析を行い、悪性中皮腫患者では特に CD8 T 細胞で CD26 陽性率の低下、細胞傷害性エフェクターT 細胞の増加､免疫チェックポイント分子の中で TIGIT と PD1 の発現陽性率の増加が認められた。

本邦で 2017 年 7 月から開始した悪性中皮腫の第 I/II 相臨床試験検体を用いて、病理組織での CD26 発現の定量・定性法の解析を行った。第 I/II 相臨床試験検体の中で、過去の病理組織の使用が許可された 8 症例について、病理組織像および R&D 社ポリクローナル抗体による免疫染色（フランスでの治験と同一の方法）による CD26 発現について検討した。その結果、組織型は上皮型が 6 例、二相型が 1 例、肉腫型が 1 例で、CD26 発現は、上皮型では 5%以下 1 例、50%2 例、70%3 例であり、二相型では、2%が 1 例、肉腫型では 50%が 1 例であった。また､FFPE 臨床検体における CD26 発現評価が可能なモノクローナル抗体 2 クローン（U16-3, U38-8）を新規開発し、上記 R&D 社抗体との比較を行ったところ、U16-3, U38-8 が R&D 社抗体と同等以上のコンパニオン診断薬になりうるものと考えられた。

悪性胸膜中皮腫は効果的な治療法が存在しないため、現在新しい治療法の開発が待たれている。一方､USP22 は新規の脱ユビキチン化酵素として発見された核蛋白である。SAGA complex 転写因子としても知られているが、様々ながんで発現しており、予後因子としても有用であると考えられている。USP22 の過剰発現が種々のがんでみられ、進行度や予後に相関していることが分かってきた。ただし、中皮腫における USP の発現や機能は、まだ不明な点が多いのが現状だ。そこで p21 を介した CD26 による細胞周期調節と関連し、中皮腫における増殖と進展における USP22 の役割を調べた。その結果、USP22 蛋白は中皮腫で発現し、USP22 ノックダ

ウンはCD26ノックダウンと同様、中皮腫の増殖を阻害することがわかった。また両者を同時にノックダウンすると、その効果がさらに増強した。しかも、USP22とCD26の発現は相関し、ヒト化CD26単クローン抗体処理はCD26を通じてUSP22のレベルを下げ、結果的にヒストンH2AとP21のユビキチン化を増加させた。したがってこれらの結果により、USP22は中皮腫治療における新しい標的になりうるとともに予後予測バイオマーカーとなる可能性も示唆され、悪性中皮腫のUSP22発現と予後やCD26抗体の治療反応性についても評価したい。

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A. 研究目的

悪性胸膜中皮腫は現時点で効果的な治療法はなく、予後は極めて不良で労災疾病行政上も大きな問題であり、有効な新規治療法の確立は急務である。

研究代表者はCD26単クローン抗体の開発、CD26 cDNAの単離を世界に先駆けて行い(J Immunol. 1989, 1992)、抗腫瘍効果の強いヒト化CD26抗体の開発に成功した。悪性中皮腫におけるCD26発現の解析、抗体の抗腫瘍作用機構の解明に取り組み、この抗体は抗体医薬特有のADCCに加え、CD26陽性腫瘍に結合することで腫瘍の増殖抑制に働くこと、さらに近年では腫瘍免疫の促進にも働きうることを明らかにしてきた(Clin Cancer Res. 2007, 2012, PLoS One. 2013, Br J Cancer. 2014, Nat Immunol. 2015)。さらに、抗体療法の確立に不可欠な病理組織の CD26発現診断用抗体、可溶性 CD26/DPPⅣ値測定系を開発し、フランスにて治療抵抗性悪性中皮腫を中心としたFirst-in-Human第Ⅰ相臨床試験を施行した。**免疫チェックポイント阻害薬のような特記すべき有害事象もなく、有効性を示唆するデータも得られたが**(Br J Cancer. 2017)、**どの患者が CD26 抗体療法の適用となるかが課題とされた。**

そこでフランスでの臨床試験患者血清を解析し、CD26/DPPⅣ値の変動解析がSD/PDの予測バイオマーカーとなる可能性が示唆された($p<0.016$)。平成29年7月から開始した第Ⅰ相臨床試験は、平成30年3月に最終患者への投与が終了し(第1～3コホート各3例、全9例)、免疫チェックポイント阻害薬で報告されているような自己免疫疾患様の重篤な副作用が出ることなく、抗腫瘍効果としてもフランスでの第 Ⅰ 相臨床試験と同様

に有効性を強く示唆する結果が得られた(**具体的な結果はキッセイ社の臨床試験結果の公表後でないと記載は不可**)。平成 30 年 6 月から第 II 相臨床試験がスタートし、患者のリクルートも順調に進んでいる。今年度の本パートでは、(1)血清に関しては、健常者と悪性中皮腫患者の血清中サイトカイン・ケモカインの多項目解析を、(2)末梢血リンパ球に関しては、健常者と国内第 I/II 相臨床試験被験者の比較解析を行った。(3)中皮腫病理組織に関しては中皮腫の CD26 発現、病理的パラメーター（組織型、細胞増殖期率、核内 p53 発現など）と CD26 抗体の有効性との相関関係を解析する。

さて、CD26 抗体により何故細胞周期の停止が生じるのかという詳しい作用機序は不明である。脱ユビキチン酵素の USP22 は多くの腫瘍でまた発現され、高発現していると予後も悪く、stage も進行しており、さらに細胞周期の調節にも関与しているといわれている。しかし悪性中皮腫細胞における発現やその機能は不明である。そこで（4）悪性中皮腫における CD26 分子と USP22 を介した細胞周期の関係および CD26 抗体処理による影響について解析を行い、USP22 が新たな治療ターゲットになり得るか、また CD26 抗体治療における USP22 の意義について検討することを目的とした。

B. 研究方法

各分担研究報告書に著述

（倫理面への配慮）

成人健常者ならびに岡山労災病院、山口宇部医療センターの悪性胸膜中皮腫患者・良性石綿胸水患者の末梢血を用いた研究については、森本が講座責任者である順天堂大学大学院医学研究科、岸本・藤本が勤務する岡山労災病院、青江が勤務する山口宇部医療センターそれぞれの施設で、本研究を行うための研究計画書等を倫理審査委員会へ提出し、承認を得ている(順大医倫第 2018127 号、岡山労災病院 115 号、山口宇部医療センター29-21 号)。また、ヒト化 CD26 抗体の国内第 I/II 相臨床試験の被験者検体を用いたバイオマーカー探索研究については、キッセイ薬品工業株式会社内の臨床試験審査委員会、各治験実施施設内の治験審査委員会にて、試験の実施と合わせてバイオマーカー探索用採血・腫瘍組織検体の提供について協議され、実施承認を取得済みである。末梢血の提供を受ける際には、研究対象者に対する人的擁護上の配慮及び研究により研究対象者が受ける不利益、利益等の説明を行い、書面でのインフォームド・コンセントを得ている。

ヒト化 CD26 抗体および USP22 および CD26 ノックダウンによる抗腫瘍効果を評価するためのマウス実験は順天堂大学実験動物委員会の審査を受け承認されている。病理組織について免疫染色して CD26 発現を解析する研究については、埼玉医科大学の倫理委員会の審査にて承認されている（承認番号 794, 861)。

C. 研究結果

1) 健常者及び悪性中皮腫患者の血清中サイトカイン・ケモカインの多項目解析

ヒト化 CD26 抗体の予後・治療効果予測バイオマーカーを探索するために、本パートでは(1)血清および(2)末梢血リンパ球の解析を行った。まず、血清に関しては、悪性中皮腫患者に特徴的な血清中サイトカイン・ケモカインを明らかにするために、成人健常者 7

例及び岡山労災病院、山口宇部医療センターから提供を受けた CD26 抗体治療を受けていない悪性中皮腫患者 10 例の血清を用いて、Bio-Plex システムによりサイトカイン・ケモカイン 49 種類の多項目測定を行った。その結果、代表的な免疫抑制性サイトカインの一つである IL-10 とマクロファージを炎症局所に留まらせるサイトカインとして知られるマクロファージ遊走阻止因子 macrophage migration inhibitory factor (MIF)の血清中濃度が、悪性中皮腫患者では健常者よりも顕著に高く、さらに悪性中皮腫患者の中には極端に高値を示す患者もいることが示された。

また、好酸球遊走が主な機能として知られるケモカイン Eotaxin/CCL11 と代表的な炎症性サイトカインの一つである IL-1β に関しては、悪性中皮腫患者と健常者との間に群平均値では血清中濃度に大きな違いは認められないが、一部の中皮腫患者で血清中濃度が極端に高かった。IL-1 family サイトカインに属する IL-33 は、健常者も悪性中皮腫患者も個人差が大きいが、血清中濃度が顕著に高い例と低い例の両方が存在した。

2) 健常者及び悪性中皮腫患者の末梢血リンパ球のフェノタイプ解析

次に、末梢血リンパ球に関して、成人健常者 5-8 例及び国内第 I/II 相臨床試験被験者 16 例(**CD26 抗体投与を開始する前**)の末梢血を用いて、フローサイトメトリーによるフェノタイプの比較を行った。まず、末梢血 CD4 T 細胞、CD8 T 細胞における CD26 の発現を解析した。代表的な T 細胞共刺激分子である CD28 と組み合わせて CD26/CD28 の発現分布を調べる方が、より詳細な情報を得られるため、CD26/CD28 の発現分布を解析した結果、いずれの健常者も末梢血 CD4 T 細胞は大部分が $CD26^{low/int}$ $CD28^{+}$に属する結果であった。悪性中皮腫患者の CD4 T 細胞は、約半数の患者は健常者と同等の発現パターンを示したが、約半数は健常者と比較して $CD26^{nega}CD28^{nega}$ の割合が多かった。

次に、CD8 T 細胞の CD26/CD28 の発現分布を解析した結果、CD4 T 細胞とは異なった。意外なことに、ほとんどの悪性中皮腫患者の末梢血 CD8 T 細胞は、Terminal Effector を意味する $CD26^{nega}$ $CD28^{nega}$ の割合が非常に高く、健常者と比較して末梢血中の CD8 T 細胞が異常に活性化した状態にあることが示唆された。極少数例(16 例中 2 例)は、健常者と同等の発現パターンを示した。これらの結果から、悪性中皮腫患者の末梢血 T 細胞、特に CD8 T 細胞は健常者と比較して CD26 陰性の割合が高いことが示された。

次に、悪性中皮腫患者の末梢血 T 細胞のフェノタイプの解析を行った。CD25 強陽性の制御性 CD4 T 細胞の割合に関しては、悪性中皮腫患者と健常者の間に顕著な違いは見られなかった。ケモカイン CXCL10 のレセプターである CXCR3 の発現陽性率は、健常者の末梢血 CD4 T 細胞、CD8 T 細胞ともに個人差が大きいが、悪性中皮腫患者では健常者と比較して、CD4・CD8 両 T 細胞で、陽性率が低い傾向にあった。また、悪性中皮腫患者では末梢血 CD8 T 細胞中の CD28 陰性、すなわち細胞傷害性エフェクターT 細胞の割合が健常者と比較して明らかに高く、同様に CD57 陽性の割合も高かった。さらに、健常者には末梢血 CD4 T 細胞中に細胞傷害性エフェクターT 細胞(CD28 陰性 CD57 陽性)はほとんど存在しないのに対し、約半数の中皮腫患者では CD4 T 細胞中 15-50%の

割合で細胞傷害活性を有するサブセットが存在することが示された。

近年、免疫系に抑制シグナルを伝達するチェックポイント分子をブロックすることで腫瘍免疫を活性化させる免疫チェックポイント阻害薬が新たながん治療法として非常に注目されている。既に治療薬として承認されている CTLA4, PD1 に加え、現在臨床試験が行われている LAG3、その他 TIM3, TIGIT, BTLA, CD160, 2B4(CD244)、また、ATP を分解してアデノシンを産生する酵素活性を有する CD39, CD73 などがある。健常者、悪性中皮腫患者ともに末梢血 CD4 T 細胞・CD8 T 細胞に CTLA4, LAG3, CD160 はほとんど発現しておらず(陽性率 0-3%)、TIM3 と BTLA の発現陽性率も 5%未満と非常に低かった。一方、PD1, TIGIT, 2B4, CD39, CD73 は明確な発現が認められた。CD4 T 細胞の PD1 および TIGIT の発現は健常者と悪性中皮腫患者の間で大きな違いは見られなかったが、CD8 T 細胞の TIGIT の発現陽性率は中皮腫患者の方が健常者よりも高かった。また、CD8 T 細胞の PD1 発現は悪性中皮腫患者内での個人差が大きいが、発現陽性率が非常に高い患者も存在した。

3) 病理組織での CD26 発現

国内第 I 相臨床試験における悪性中皮腫 9 症例および第 II 相臨床試験における悪性中皮腫 3 症例の中で、過去の病理組織の使用が許可された 8 症例について、病理組織像および R&D 社ポリクローナル抗体による免疫染色（フランスでの治験と同一の方法）による CD26 発現について検討した。8 症例は、上皮型中皮腫 6 例、二相型中皮腫 2 例（1 例は生検ではほとんど肉腫型）であり、CD26 発現は、上皮型で、5%以下 1 例、50%2 例、70%3 例であり、二相型では、2%が 1 例、肉腫型では 50%が 1 例であった。組織型および CD26 発現とヒト化 CD26 抗体の治療効果との関係については、次年度により症例数を追加して、第 II 相臨床試験が終了した時点で解析を行う予定である。

これらの染色標本をデジタル画像とし、imageProPlus プログラムにて詳細に半定量的に発現解析を行った。その結果、CD26 は細胞膜および細胞質に 95%が、核内に 1-2%の発現が認められた。現在、これらのデータベースを元に臨床パラメーターとの相関について検討している。

また FFPE 臨床検体における CD26 発現評価が可能な新規開発したモノクローナル抗体 2 クローン（U16-3, U38-8）と上記 R&D 社ポリクローナル抗体との比較を行った。その結果、中皮腫 69 症例において、抗体 3 種類での陽性一致率は、94%であり、陽性率 10%以上での不一致例は 0 例であった。また抗体 3 種類における陽性パターン（細胞膜、細胞質）は、ほぼ完全に一致していたが、陽性例における S/N 比は、U38-8 がもっとも優れていた。この結果は、新規モノクローナル抗体 U16-3, U38-8 は、R&D 社ポリクローナル抗体と同等以上のコンパニオン診断薬になりうるものと考えられた。

4) 中皮腫臨床サンプルにおける USP22 の発現と、USP22 ノックダウンの細胞増殖阻害効果

USP22 はさまざまながんで発現が報告されているが、悪性中皮腫ではまだ詳しくは調べられておらず、まずそれについて実験を行った。その結果、USP22 は悪性中皮腫の全ての組織型（上皮型、肉腫型、混合型）で発現がみられた。上皮型では 26 例中 21 例(81%)で陽性だったのに対し、肉腫型では 5 例中 3 例(60%)、混合型では 12 例中 7 例(58%)が陽

性だった。

次に中皮腫細胞株でもUSP22の発現を調べたが、MESO1株とJMN株では核と細胞質の両方でUSP22の発現が見られた。

USP22のshRNAノックダウン実験を行ったところ、その増殖が抑制された。しかもその細胞は、マウス移植実験でも腫瘍増殖が抑制され、生存も延長した。これらの結果からUSP22のノックダウンは中皮腫の増殖と成長を抑制することが示された。

5) USP22とCD26のダブルノックダウンは、いずれか一方のノックダウンより、中皮腫細胞の増殖を強く阻害する

以前、CD26分子のノックダウンが中皮腫の増殖と浸潤を抑制することを報告している。一方、CD26及びUSP22ともに癌幹細胞特性に関連していると言われている。そこでUSP22とCD26が協調効果を示すかどうかを調べた。MESO1株はCD26+USP22+とCD26- USP22+分画を含んでいるのに対し、JMN株はほとんどCD26+USP22+分画細胞だけから成っていた。

次にMESO1株をCD26+とCD26-に分離して解析したところ、CD26+細胞の方がCD26-細胞よりもin vitroで培養した時の細胞増殖およびin vivoでマウスに皮下移植した時の増殖が速かった。これからの結果から、CD26はUSP22と協調して増殖を促進していることが示唆された。

CD26発現減少がCDKIのp21発現増強を介した細胞周期調節に関わっていることを報告し、USP22もCDKIのp21を標的にしていることを明らかにした。そこでCD26とUSP22ノックダウン細胞の細胞周期解析を行ったところ、G1/S arrestが生じていたことが判明した。これらのことから、CD26とUSP22は細胞周期に影響することで、阻害効果を示すことがわかった。また両者のノックダウンでは、その効果が増強された。

悪性中皮腫患者検体において、CD26陽性の中皮腫のうちUSP22も発現している症例は上皮型で71%、肉腫型で33%、混合型で71%だったが、CD26とUSP22が共陽性の中皮腫患者の方が予後不良である傾向が見られており、CD26とUSP22が協調して中皮腫の悪性化に関与していることが示唆された。

6) ヒト化CD26抗体はCD26とUSP22を介して中皮腫細胞の増殖を抑制する

上記の実験で、CD26とUSP22に相互作用が示唆されたため、それぞれの発現がお互いの発現に影響するかどうかを調べた。その結果、USP22をノックダウンするとCD26発現も減少し、USP22を過剰発現させるとCD26発現も増加することが示された。

USP22は脱ユビキチン化酵素活性を持つため、次にUSP22発現に関連したCD26のユビキチン化状態を調べた。その結果、USP22の発現が減少すればCD26のユビキチン化が増加していることが示された。このことから、USP22+CD26+中皮腫細胞では、USP22がCD26の発現を調節していることが示唆された。

CD26は主に細胞表面に発現しているのに対して、USP22はSAGA転写因子コファクター複合体の一つであり核に局在している。我々は最近、細胞表面上のCD26分子にヒト化CD26抗体が結合すると、CD26とヒト化CD26抗体が複合体を形成したまま細胞内、さらには核に移行し、中皮腫細胞の増殖抑制に作用することを示したが、この

核に移行した CD26 が USP22 に作用し、USP22 の発現減少と p21 の発現上昇を引き起こすと推測した。

ヒト化 CD26 抗体の用量依存的に CD26 と USP22 は複合体を形成し、しかもそれは、核内 USP22 量の減少をもたらしたが、別の SAGA 複合体である GCN5 には影響しなかった。

USP22 の鍵となる機能は、p21 発現を調節するヒストン H2A の脱ユビキチン化だが、我々はさらにこの点を検討した。その結果、ヒト化 CD26 抗体処理はヒストン H2A の脱ユビキチン化と p21 の発現レベルを増強させることがわかった。同様の結果は USP22 のノックダウンでも得られた。しかもヒト化 CD26 抗体処理は、用量依存的に細胞増殖を阻害し、G1/S arrest を引き起こした。

これらの結果は、ヒト化 CD26 抗体処理が核内の USP22 を減少させることで、中皮腫細胞の増殖を抑制することを強く示唆している。

今回の研究により CD26 抗体処理による細胞周期停止および CDKI p21 の発現誘導の分子メカニズムを明らかにでき、USP22 は新たな治療ターゲットになり得、また USP22 の悪性中皮腫での発現評価はヒト化 CD26 抗体の治療反応性予測などのバイオマーカーとなる可能性も示唆された。

D. 考察

ヒト化 CD26 抗体の予後・治療効果予測バイオマーカーを開発するために、悪性中皮腫患者の血清中サイトカイン・ケモカインの多項目解析と、末梢血リンパ球の CD26 発現およびフェノタイプ解析、免疫チェックポイント分子の発現解析を主に行った。

我々は末梢血 CD4 T 細胞を用いて、強い CD26 共刺激シグナルが伝達すると転写因子 EGR2 の顕著な発現上昇と IL-10 の高産生が誘導されることを報告している(J Immunol. 2015)。悪性中皮腫患者の血清中 IL-10 濃度は健常者よりも顕著に高く、さらに中皮腫患者の中には極端に高値を示す患者もいたことは、非常に興味深い結果である。悪性中皮腫細胞自身にも CD26 のリガンドである caveolin-1 が発現しているため、中皮腫周囲に浸潤した T 細胞に CD26 共刺激が伝達して IL-10 の高産生を誘導している可能性が考えられる。ヒト化 CD26 抗体は CD26 に結合して、CD26 と caveolin-1 との結合阻害にも働くことから、免疫抑制性サイトカインである IL-10 の産生抑制に働くことが期待される。

近年、マウス担癌モデルにおいて、DPPIV inhibitor (Sitagliptin)をエサと一緒にマウスに食べさせることで、腫瘍免疫が増強し、腫瘍サイズが縮小することが報告された(Nat Immunol. 2015)。そのメカニズムとして、がん微小環境から産生されるケモカインの中で DPPIV 酵素の基質の一つである CXCL10 に着目し、本来は産生された CXCL10 が DPPIV 酵素による切断を受けると活性が低下するのに対し、DPPIV inhibitor を摂取することで、CXCL10 の活性が維持され、そのレセプターである CXCR3 陽性の CD4 T 細胞、CD8 T 細胞、NK 細胞ががん細胞周囲により集積するようになり、免疫細胞が腫瘍をより攻撃しやすくなることを示している。フランスでのヒト化 CD26 抗体の第 I 相臨床試験の結果から、CD26 抗体を投与すると容量依存的に血清中の可溶性 CD26 量および DPPIV 酵素活性

が低下するデータを得ている(Br J Cancer. 2017)。このことから、CD26 抗体を投与した場合においても、DPPIV によって切断されるケモカインの量が減少し、ケモカインの活性維持、がん細胞周囲に集積する免疫細胞数の増加が起こる可能性が考えられる。悪性中皮腫患者の末梢血 T 細胞の CXCR3 の発現陽性率は CD4 T 細胞、CD8 T 細胞ともに、健常者と比較して低い傾向にあった。しかしながら、一部の中皮腫患者では CXCR3 の発現陽性率が高いことから、これらの CXCR3 の発現率が高い患者と CD26 抗体の治療有効性との関係性について今後着目したい。

がん微小環境に浸潤した T 細胞は、がん細胞自身が産生する免疫抑制性因子や PD1/PDL1・PDL2、CTLA4/CD80・CD86 に代表される免疫チェックポイント分子シグナルによって、エフェクター機能が抑制されていることが様々ながん種で報告されている。悪性中皮腫患者では末梢血 CD8 T 細胞中の細胞傷害性エフェクターT 細胞(CD28 陰性 CD57 陽性)の割合が健常者と比較して明らかに高く、さらに CD4 T 細胞においても細胞傷害活性を有するサブセットが存在することが示された。このような perforin と granzyme を発現する細胞傷害性エフェクターT 細胞は本来、ウイルスに感染した細胞やがん細胞を体内から除去するために働く細胞だが、悪性中皮腫患者の末梢血中にどうして細胞傷害活性を有する T 細胞がこれほど多く存在するのか、何の抗原に対して活性化された T 細胞なのかは興味深い疑問である。免疫チェックポイント分子の中で、悪性中皮腫患者の末梢血 CD8 T 細胞は TIGIT の発現陽性率が健常者よりも明白に高く、また PD1 の発現も高い傾向が見られた。末梢血中の細胞傷害性エフェクターT 細胞の割合や CD8 T 細胞の TIGIT 及び PD1 の発現と CD26 抗体の治療有効性との相関関係について次年度に解析を行う。

病理組織について、悪性中皮腫における CD26 発現について免疫染色にて評価し、組織型とともに発現パターン（細胞局在、陽性率、陽性強度などの各種パラメーター）を詳細に定量評価することにより、抗体療法の効果や予後などとの関連性あるいは臨床パラメーターを検証する基礎を構築していくことが重要と考える。

USP22 の研究結果はヒト化 CD26 抗体処理が核内の USP22 を減少させることで、中皮腫細胞の増殖を抑制することを強く示唆している。さらに、USP22 による細胞周期調節と中皮腫細胞の増殖における、ヒト化 CD26 抗体の影響のモデルであるが、恒常的な USP22 の高発現はヒストン H2A の脱ユビキチン化を安定化し、ヘテロクロマチンをサイレンシングすることで p21 発現を抑制し、その結果、腫瘍の異常増殖を引き起こす。一方、ヒト化 CD26 抗体処理による CD26 の核移行は核内での CD26-USP22 複合体形成を引き起こし、核内の USP22 量を減少させる。これにより、ヘテロクロマチンサイレンシングが解除され、CDKI p21 を含む特定の遺伝子を活性化し、腫瘍の増殖を抑制すると考えられる。

今回の研究により CD26 抗体処理による細胞周期停止および CDKI p21 の発現誘導の分子メカニズムを明らかにでき、USP22 は新たな治療ターゲットになり得、また USP22 の悪性中皮腫での発現評価はヒト化 CD26 抗体の治療反応性予測などのバイオマーカーとなる可能性も示唆された。

E. 結論

1) 悪性中皮腫患者と健常者の血清中サイトカイン・ケモカインの多項目解析を行い、ヒト化 CD26 抗体の予後・治療効果予測バイオマーカー候補として、IL-10, MIF, Eotaxin/CCL11, IL-1β, IL-33 を見出した。また、国内第 I/II 相臨床試験患者の末梢血リンパ球のフェノタイプ解析を行い、悪性中皮腫患者では CXCR3 の発現が低下している傾向が見られ、また、特に CD8 T 細胞で CD26 陽性率の低下、細胞傷害性エフェクターT 細胞の増加、免疫チェックポイント分子の中で TIGIT と PD1 の発現陽性率の増加が認められた。

2) 臨床試験における悪性中皮腫検体における CD26 発現評価を行い、半定量的解析法を確立した。また FFPE 検体で使用可能な新規モノクローナル抗体 U16-3, U38-8 の開発を通じて、CD26 抗体療法におけるコンパニオン診断薬に有用であることを見出した。

3) USP22 の抑制は中皮腫細胞の増殖を抑制し、ヒト化 CD26 単クローン抗体処理は表面 CD26 を内在化させ、UPS22 と物理的に接触させ、CDKI p21 の発現上昇で腫瘍増殖を抑制する。その一方で、ヒト化 CD26 抗体による CD26 標的化は USP22 を減少させることが可能なため、将来的には中皮腫のみならずさまざまな CD26 陽性がんで、有用なアプローチになり得、またその悪性中皮腫での発現評価はヒト化 CD26 抗体の治療反応予測のバイオマーカーとなる可能性が示唆された。

F. 健康危険情報

現時点では特記すべき健康危険情報はない。

G. 今後の展望

次年度は、今年度に引き続き国内第Ⅱ相臨床試験被験者や対照症例の解析検体数をさらに増やすとともに、血清中可溶性 CD26 濃度/DPPIV 酵素活性値に加え、上記のバイオマーカー候補の発現と、ヒト化 CD26 抗体の治療有効性との相関関係を解析し、予後・治療効果バイオマーカーを同定する。

今年度と継続して国内第Ⅱ相臨床試験の悪性中皮腫の病理組織像および CD26 発現を解析し、組織型や CD26 発現の細胞局在、陽性率、陽性強度、細胞増殖期率など中皮腫の様々な病理学的パラメーターを解析し、ヒト化 CD26 抗体の有効性評価項目との相関を統計学的に解析することで、CD26 抗体の予後・治療効果予測バイオマーカーとなりうるかどうかを検討する。

悪性中皮腫組織細胞における USP22 の発現評価は悪性中皮腫における予後およびヒト化 CD26 抗体の治療反応性予測のバイオマーカーとなり得るかどうかについても今後解析を行う予定である。

H.研究発表

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I. 知的財産権の出願・登録状況（予定を含む）

1.　特許取得

1）名称：免疫チェックポイント阻害剤
発明者：大沼圭、森本幾夫、波多野良
出願日：2019 年 1 月 15 日（PCT 出願）
出願番号：特願 2019-04480
出願人：学校法人順天堂

2）名称：抗ヒト CD26 モノクローナル抗体
発明人: 森本幾夫､波多野良､山田健人、大沼圭.
出願日　：2018 年 3 月 16 日(PCT 出願)
出願番号：　特願 2018-049308
出願人：(権利者）学校法人順天堂、学校法人埼玉医科大学
（共同出願）

2.　実用新案登録
なし

3.　その他
なし

Ⅱ．　分担研究報告

労災疾病臨床研究事業費補助金
分担研究報告書
ヒト化 CD26 抗体の予後・治療効果予測バイオマーカーの探索：血清および末梢血リンパ球の解析結果について

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研究要旨

悪性胸膜中皮腫はアスベストばく露によって起こる難治性悪性腫瘍であり、現時点で満足できる治療法はなく、新たな治療法の確立が望まれる。われわれは、新規治療標的分子として悪性胸膜中皮腫に発現する CD26 に着目し、ヒト化 CD26 抗体を開発しフランスにて第 I 相臨床試験を行った。安全性が確認されるとともに治療薬としての有効性を示唆する結果も得られ、平成 29 年 7 月から国内でも第 I/II 相臨床試験を開始した。平成 30 年 3 月に第 I 相臨床試験最終患者への投与が終了し、同年 6 月からスタートした第 II 相臨床試験も患者のリクルートが順調に進んでいる。今年度は悪性中皮腫患者と健常者の血清中サイトカイン・ケモカインの多項目解析を行い、一部の悪性中皮腫患者では IL-10, MIF, Eotaxin/CCL11, IL-1β の濃度が顕著に高いことを見出した。また、国内第 I/II 相臨床試験患者の末梢血リンパ球のフェノタイプ解析を行い、悪性中皮腫患者では特に CD8 T 細胞で CD26 陽性率の低下、細胞傷害性エフェクターT 細胞の増加、免疫チェックポイント分子の中で TIGIT と PD1 の発現陽性率の増加が認められた。次年度に、悪性中皮腫患者の血清および末梢血リンパ球のこれらバイオマーカー候補の発現と、ヒト化 CD26 抗体の治療有効性との相関関係を解析し、予後・治療効果バイオマーカーを同定する。

A. 研究目的

悪性胸膜中皮腫はアスベストばく露によって起こる胸膜中皮由来の難治性悪性腫瘍である。アスベストばく露から発症までの潜伏期間は 30-50 年とされ、日本を含めアジアやヨーロッパなど世界規模で患者数は今

後ますます増加すると考えられている。予後は極めて悪く、手術療法、化学療法、放射線療法などが行われるが、いずれも満足できる治療成績ではなく、新たな治療法の確立が望まれる。われわれは、新規治療標的分子として悪性中皮腫細胞に発現する CD26 に着目し、ヒト化 CD26 抗体を開発してフランスにて悪性中皮腫を中心に First-in-Human 第 I 相臨床試験を行った。Infusion reaction (急性輸注反応)を除いて特記すべき副作用もなく、安全性が確認されるとともに、抗がん剤抵抗性の悪性中皮腫患者 19 例中 10 例が modified RESIST 評価で Stable Disease (SD)となり、そのうち 5 例は 6 ヶ月以上、最長で投与を開始してから 399 日 SD が持続し、治療薬としての有効性を示唆する結果も得られた(Br J Cancer. 2017)。

このフランスでの第 I 相臨床試験の結果を受け、いかなる患者が CD26 抗体療法の適用となるのか、CD26 抗体療法の治療効果や予後を予測できるバイオマーカーの探索が課題として挙げられた。このことは、本抗体療法がより安全かつ効果的に行われるうえで極めて重要であり、かつ、抗体療法適用患者を適切に選択できれば期待していた治療効果が得られない患者にまで高額な抗体医薬の医療費負担を強いることがなくなる。

我々はこれまでにヒト化 CD26 抗体の抗腫瘍作用メカニズムとして、抗体医薬特有の抗体依存性細胞傷害(ADCC)活性・補体依存性細胞傷害(CDC)活性に加え、CD26 陽性腫瘍に抗体が結合することによる直接的な作用があることを明らかにしてきた。がん細胞の細胞膜上の CD26 にヒト化 CD26 抗体が結合すると、cyclin dependent kinase inhibitor である p21 や p27 の発現が上昇し cell cycle arrest を起こさせること(Clin Cancer Res. 2001, Immunology. 2002, Clin Cancer Res. 2007, Cancer Cell Int. 2016)、CD26 抗体と CD26 の複合体が細胞膜から細胞質、さらに核内へと移行し、RNA polymerase II のサブユニットである POLR2A 遺伝子の転写領域下流に結合することで POLA2A の転写を抑制し増殖抑制に働くことを明らかにした(PLoS One. 2013)。

また、CD26 はヒト T 細胞に活性化シグナルを伝達する T 細胞共刺激分子でもあり、ヒト化 CD26 抗体は CD26 のリガンドである caveolin-1 と CD26 との結合、つまりは T 細胞への CD26 共刺激シグナルの伝達をブロックする。また、CD26 の機能の一つに dipeptidyl peptidase IV (DPPIV)酵素活性があり、N 末から 2 番目にプロリンまたはアラニンを有するペプチドの 2 アミノ酸を切断する。生体内で様々な生理活性物質がその基質となることが知られているが、いくつかのケモカインも DPPIV による切断を受けその細胞遊走活性が不活性化される。ヒト化 CD26 抗体は DPPIV 酵素活性自体に直接は影響しないが、フランスでの第 I 相臨床試験の結果から CD26 抗体の投与により血中の可溶性 CD26 の量が顕著に低下し、DPPIV 酵素活性も同様に低下することが示されている(Br J Cancer. 2017)。DPPIV 酵素活性の低下により IP-10(CXCL10)などのケモカインの切断と不活性化が抑えられ、免疫細胞が腫瘍組織に遊走しやすくなる可能性が考えられる(Nat Immunol. 2015)。これらの知見から、CD26 抗体は免疫系にも影響する可能性が強く示唆される。

そこで、これまでに我々が解明してきた CD26 抗体の抗腫瘍作用メカニズムに基づ

き、本抗体の予後・治療効果予測バイオマーカーを同定するために、平成 29 年 7 月から本邦で開始した治療抵抗性(標準治療で Progressive Disease (PD))の悪性胸膜中皮腫に対するヒト化 CD26 抗体の第 I/II 相臨床試験被験者の(1)中皮腫病理組織、(2)血清、(3)末梢血リンパ球の解析を行う。(1)中皮腫病理組織に関しては、中皮腫の CD26 発現、病理学的パラメーター(組織型、細胞増殖期率、核内 p53 発現、脈管侵襲など)と CD26 抗体の有効性との相関関係を解析する。(2)血清に関しては、フランスでの第 I 相臨床試験被験者の血清を解析した結果、CD26 抗体投与による血清中可溶性 CD26 値および DPPIV 酵素活性の変動解析は、CD26 抗体の治療効果予測バイオマーカーとなる可能性が示唆され(p<0.016)、国内臨床試験被験者の検体を用いて検証を行う。また、前に述べたように CD26 抗体は T 細胞への CD26 共刺激シグナルの伝達阻害ならびに DPPIV 酵素活性の低下にも作用するため、免疫系にも何かしらの影響があることが予想される。そのため、CD26/DPPIV と関連するサイトカイン・ケモカインの多項目解析を行い、悪性中皮腫に特徴的なサイトカイン・ケモカインの解明、さらに CD26 抗体投与による血清中濃度の変動を解析し、CD26 抗体の有効性との相関関係を解析する。(3)末梢血リンパ球に関しては、末梢血中の CD4 T 細胞・CD8 T 細胞の細胞数・割合、CD26 および CXCR3(ケモカイン CXCL10 の受容体)の発現、Perforin や Granzyme といったがん細胞や感染細胞を除去するための細胞傷害活性を有するエフェクターT 細胞の細胞数・割合、近年着目されている代表的な免疫チェックポイント分子の発現、CD25 強陽性の制御性 CD4 T 細胞の細胞数・割合を解析し、CD26 抗体の有効性との相関関係を解析する。

平成 29 年 7 月から開始した第 I 相臨床試験は、平成 30 年 3 月に最終患者への投与が終了し(第 1～3 コホート各 3 例、全 9 例)、免疫チェックポイント阻害薬で報告されているような自己免疫疾患様の重篤な副作用が出ることなく、抗腫瘍効果としてもフランスでの第 I 相臨床試験と同様に有効性を強く示唆する結果が得られた(**具体的な結果はキッセイ社の臨床試験結果の公表後でないと記載は不可**)。平成 30 年 6 月から第 II 相臨床試験がスタートし、患者のリクルートも順調に進んでいる。今年度の本パートでは、(2)血清に関しては、健常者と悪性中皮腫患者の血清中サイトカイン・ケモカインの多項目解析を、(3)末梢血リンパ球に関しては、健常者と国内第 I/II 相臨床試験被験者の比較解析を行った。

B. 研究方法

1) Bio-Plex マルチプレックスアッセイ

成人健常者および岡山労災病院、山口宇部医療センターから提供を受けた悪性胸膜中皮腫患者・良性石綿胸水患者の血清中サイトカイン・ケモカイン濃度を Bio-Plex マルチプレックスシステムにより測定した。Bio-Plex Pro Human Chemokine 40-Plex panel および Bio-Plex Pro Human Th17 Cytokine 15-Plex panel (Bio-Rad)を用いて、付属のプロトコルに従い Bio-Plex system (Bio-Rad)で測定を行い、得られたデータを Bio-Plex Manager (Bio-Rad)で解析した。

2) 抗体と試薬

Flow cytometry には下記のヒト抗原特異抗体を用いた。PE-labeled anti-CD26 mAb (clone M-A261)は BD Biosciences から購入した。PE/Cy7-labeled anti-KLRG1 mAb (clone 13F12F2), PE/Cy7-labeled anti-TIGIT mAb (clone MBSA43), APC-labeled anti-KLRG1 mAb (clone 13F12F2), APC-labeled anti-TIGIT mAb (clone MBSA43) 及び APC-labeled anti-LAG3 mAb (clone 3DS223H) は eBioscience から購入した。FITC-labeled anti-CD4 mAb (clone RPA-T4), FITC-labeled anti-CD8 mAb (clone HIT8a), PE/Cy7-labeled anti-CD25 mAb (clone clone M-A251), PE/Cy7-labeled anti-CD28 mAb (clone clone CD28.2), PE/Cy7-labeled anti-CD56 mAb (clone 5.1H11), PE/Cy7-labeled anti-PD1 mAb (clone EH12.2H7), APC-labeled anti-CD28 mAb (clone clone CD28.2), APC-labeled anti-CD57 mAb (clone clone HCD57), APC-labeled anti-CXCR3 mAb (clone G025H7), APC-labeled anti-TRAIL mAb (clone RIK-2), APC-labeled anti-BTLA mAb (clone MIH26), APC-labeled anti-PD1 mAb (clone EH12.2H7), APC-labeled anti-Tim3 mAb (clone F38-2E2)及び抗体の非特異的な結合をブロックするための Human TruStain FcX は BioLegend から購入した。

3) フローサイトメトリー

成人健常者および国内第 I/II 相臨床試験の被験者から提供を受けた末梢血を、BD Pharm Lyse Lysing Buffer (BD Biosciences)にて溶血処理を行い、洗浄した後、Human TruStain FcX を添加し、続いて蛍光色素で標識した各種抗体を添加して細胞膜上の目的タンパク質の染色を行った。FACSCalibur (BD Biosciences)で測定を行い、得られたデータを FlowJo (BD Biosciences)で解析した。

(倫理面への配慮)

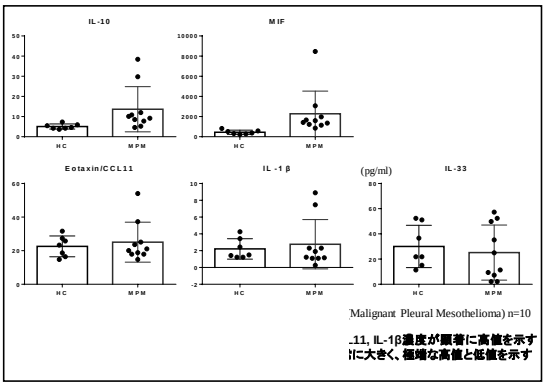
成人健常者ならびに岡山労災病院、山口宇部医療センターの悪性胸膜中皮腫患者・良性石綿胸水患者の末梢血を用いた研究については、森本が講座責任者である順天堂大学大学院医学研究科、岸本・藤本が勤務する岡山労災病院、青江が勤務する山口宇部医療センターそれぞれの施設で、本研究を行うための研究計画書等を倫理審査委員会へ提出し、承認を得ている(順大医倫第 2018127 号、岡山労災病院 115 号、山口宇部医療センター29-21 号)。また、ヒト化 CD26 抗体の国内第 I/II 相臨床試験の被験者検体を用いたバイオマーカー探索研究については、キッセイ薬品工業株式会社内の臨床試験審査委員会、各治験実施施設内の治験審査委員会にて、試験の実施と合わせてバイオマーカー探索用採血・腫瘍組織検体の提供について協議され、実施承認を取得済みである。末梢血の提供を受ける際には、研究対象者に対する人的擁護上の配慮及び研究により研究対象者が受ける不利益、利益等の説明を行い、書面でのインフォームド・コンセントを得ている。

C. 研究結果

1) 健常者及び悪性中皮腫患者の血清中サイトカイン・ケモカインの多項目解析

ヒト化 CD26 抗体の予後・治療効果予測バイオマーカーを探索するために、本パート

では(2)血清および(3)末梢血リンパ球の解析を行った。まず、血清に関しては、悪性中皮腫患者に特徴的な血清中サイトカイン・ケモカインを明らかにするために、成人健常者 7 例及び岡山労災病院、山口宇部医療センターから提供を受けた CD26 抗体治療を受けていない悪性中皮腫患者 10 例の血清を用いて、Bio-Plex システムによりサイトカイン・ケモカイン 49 種類の多項目測定を行った。その結果、代表的な免疫抑制性サイトカインの一つである IL-10 とマクロファージを炎症局所に留まらせるサイトカインとして知られるマクロファージ遊走阻止因子 macrophage migration inhibitory factor (MIF)の血清中濃度が、悪性中皮腫患者では健常者よりも顕著に高く、さらに悪性中皮腫患者の中には極端に高値を示す患者もいることが示された(図 1)。



また、好酸球遊走が主な機能として知られるケモカイン Eotaxin/CCL11 と代表的な炎症性サイトカインの一つである IL-1β に関しては、悪性中皮腫患者と健常者との間に群平均値では血清中濃度に大きな違いは認められないが、一部の中皮腫患者で血清中濃度が極端に高かった(図 1)。IL-1 family サイトカインに属する IL-33 は、健常者も悪性中皮腫患者も個人差が大きいが、血清中濃度が顕著に高い例と低い例の両方が存在した(図 1)。今後、国内第 I/II 相臨床試験検体を用いて、ヒト化 CD26 抗体投与前のこれらの濃度、さらには抗体投与によるこれらの濃度の変動と CD26 抗体の予後・治療有効性との関係性を明らかにする。

血清中の可溶性CD26濃度/DPPIV酵素活性値の解析に関しては、**キッセイ社の臨床試験結果の公表後でないと記載ができない**が、これらに関しても引き続き、国内第 I/II 相臨床試験検体を用いて、CD26 抗体の予後・治療有効性との関係性を解析していく。

2) 健常者及び悪性中皮腫患者の末梢血リンパ球のフェノタイプ解析

次に、末梢血リンパ球に関して、成人健常者 5-8 例及び国内第 I/II 相臨床試験被験者 16 例(**CD26 抗体投与を開始する前**)の末梢血を用いて、フローサイトメトリーによるフェノタイプの比較を行った。まず、末梢血 CD4 T 細胞、CD8 T 細胞における CD26 の発現を解析した。CD26 は健常者の末梢血 CD4 T 細胞、CD8 T 細胞ともに、CD26 高発現($CD26^{high}$)・CD26 低発現($CD26^{low/int}$)・CD26 陰性($CD26^{nega}$)の三相性の特徴的な発現パターンを示し、それぞれが Naive・Central Memory・Effector Memory・Terminal Effector といった T 細胞の分化段階と密接に関係している(Immunology. 2013)。代表的な T 細胞共刺激分子である CD28 と組み合わせて CD26/CD28 の発現分布を調べる方が、より詳細な情報を得られるため、CD26/CD28 の発現分布を解析した結果、図 2 に示すように、いずれの健常者も末梢血 CD4 T 細胞は大部分が $CD26^{low/int}$ $CD28^{+}$に属する結果であった。悪性中皮腫

患者の CD4 T 細胞は、約半数の患者は健常者と同等の発現パターンを示したが(図 2 中 Type A)、約半数は健常者と比較して $CD26^{nega}CD28^{nega}$ の割合が多かった(図 2 中 Type B)。

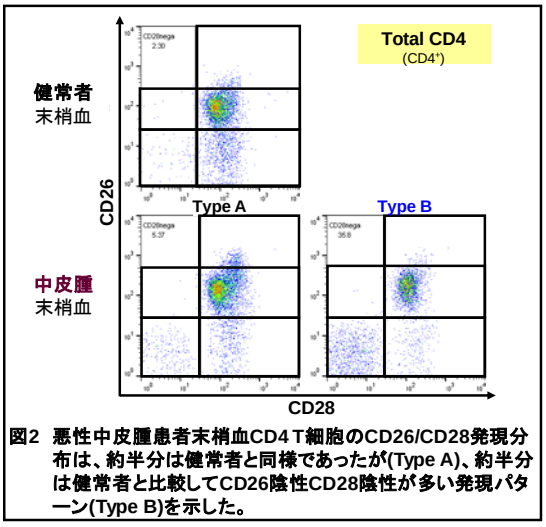


図2 悪性中皮腫患者末梢血CD4 T細胞のCD26/CD28発現分布は、約半分は健常者と同様であったが(Type A)、約半分は健常者と比較してCD26陰性CD28陰性が多い発現パターン(Type B)を示した。

次に、CD8 T 細胞の CD26/CD28 の発現分布を解析した結果、CD4 T 細胞とは異なり CD8 T 細胞は健常者でも Naive・Memory・Terminal Effector の割合の個人差は大きいが、図 3 に代表的な末梢血 CD8 T 細胞の発現パターンを示す。主に 4 つのサブセットに分類することができ、$CD26^{high}CD28^{+}$は早期 Effector Memory、$CD26^{low/int}CD28^{+}$は Naive、$CD26^{nega}CD28^{+}$は全ての分化段階の混合、$CD26^{nega}CD28^{nega}$は主に Terminal Effector に属することを、以前報告した(Immunology. 2013)。意外なことに、ほとんどの悪性中皮腫患者の末梢血 CD8 T 細胞は、Terminal Effector を意味する $CD26^{nega}$ $CD28^{nega}$ の割合が非常に高く、健常者と比較して末梢血中の CD8 T 細胞が異常に活性化した状態にあることが示唆された(図 3 中 Type B)。極少数例(16 例中 2 例)は、健常者と同等の発現パターンを示した(図 3 中 Type A)。これらの結果から、悪性中皮腫患者の末梢血 T 細胞、特に CD8 T 細胞は健常者と比較して CD26 陰性の割合が高いことが示された。

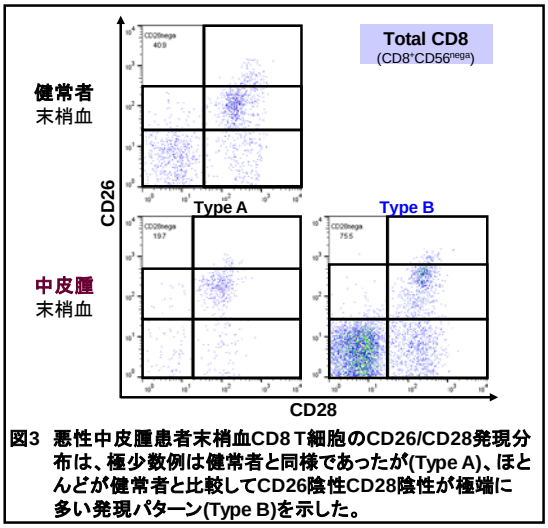
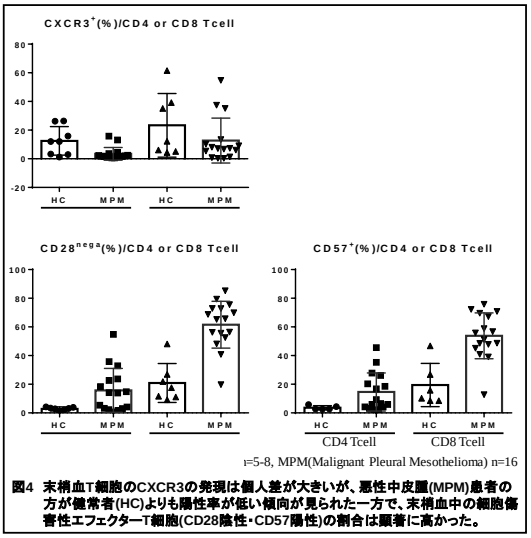


図3 悪性中皮腫患者末梢血CD8 T細胞のCD26/CD28発現分布は、極少数例は健常者と同様であったが(Type A)、ほとんどが健常者と比較してCD26陰性CD28陰性が極端に多い発現パターン(Type B)を示した。

次に、悪性中皮腫患者の末梢血 T 細胞のフェノタイプの解析を行った。CD25 強陽性の制御性 CD4 T 細胞の割合に関しては、悪性中皮腫患者と健常者の間に顕著な違いは見られなかった(データ未掲載)。ケモカイン CXCL10 のレセプターである CXCR3 の発現陽性率は、健常者の末梢血 CD4 T 細胞、CD8 T 細胞ともに個人差が大きいが、悪性中皮腫患者では健常者と比較して、CD4・CD8 両 T 細胞で、陽性率が低い傾向にあった(図 4)。また、図 3 で示したように、悪性中皮腫患者では末梢血 CD8 T 細胞中の CD28 陰性、すなわち細胞傷害性エフェクターT 細胞の割合が健常者と比較して明らかに高く、同様に CD57 陽性の割合も高かった(図 4)。さらに、健常者には末梢血 CD4 T 細胞中に細胞傷害性エフェクターT 細胞(CD28 陰性 CD57 陽性)はほとんど存在しないのに対し、約半数の中皮腫患者では CD4

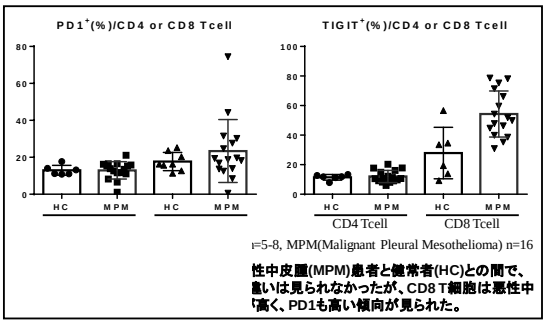
T 細胞中 15-50%の割合で細胞傷害活性を有するサブセットが存在することが示された(図 4)。



n=5-8, MPM(Malignant Pleural Mesothelioma) n=16

図4 末梢血T細胞のCXCR3の発現は個人差が大きいが、悪性中皮腫(MPM)患者の方が健常者(HC)よりも陽性率が低い傾向が見られた一方で、末梢血中の細胞傷害性エフェクターT細胞(CD28陰性・CD57陽性)の割合は顕著に高かった。

近年、免疫系に抑制シグナルを伝達するチェックポイント分子をブロックすることで腫瘍免疫を活性化させる免疫チェックポイント阻害薬が新たながん治療法として非常に注目されている。多くの免疫チェックポイント分子の総説で取りあげられ着目されている免疫チェックポイント分子として、既に治療薬として承認されている CTLA4, PD1 に加え、現在臨床試験が行われている LAG3、その他 TIM3, TIGIT, BTLA, CD160, 2B4(CD244)、また、ATP を分解してアデノシンを産生する酵素活性を有する CD39, CD73 などがある。健常者、悪性中皮腫患者ともに末梢血 CD4 T 細胞・CD8 T 細胞に CTLA4, LAG3, CD160 はほとんど発現しておらず(陽性率 0-3%)、TIM3 と BTLA の発現陽性率も 5%未満と非常に低かった(データ未掲載)。一方、PD1, TIGIT, 2B4, CD39, CD73 は明確な発現が認められた(データ未掲載)。CD4 T 細胞の PD1 および TIGIT の発現は健常者と悪性中皮腫患者の間で大きな違いは見られなかったが、CD8 T 細胞の TIGIT の発現陽性率は中皮腫患者の方が健常者よりも高かった(図 5)。また、CD8 T 細胞の PD1 発現は悪性中皮腫患者内での個人差が大きいが、発現陽性率が非常に高い患者も存在した(図 5)。今後、末梢血 CD4・CD8 両 T 細胞の CXCR3 発現、細胞傷害性エフェクターT 細胞の割合、CD8 T 細胞の PD1 と TIGIT の陽性率に着目し、CD26 抗体の治療有効性との関係性を明らかにする。



n=5-8, MPM(Malignant Pleural Mesothelioma) n=16

性中皮腫(MPM)患者と健常者(HC)との間で、違いは見られなかったが、CD8 T細胞は悪性中皮腫患者で高く、PD1も高い傾向が見られた。

D. 考察

ヒト化 CD26 抗体の予後・治療効果予測バイオマーカーを開発するために、今年度の本パートでは、悪性中皮腫患者の血清中サイトカイン・ケモカインの多項目解析と、末梢血リンパ球の CD26 発現およびフェノタイプ解析、免疫チェックポイント分子の発現解析を主に行った。

我々は健常者の末梢血 CD4 T 細胞を用いて、強い CD26 共刺激シグナルが伝達すると転写因子 EGR2 の顕著な発現上昇と IL-10 の高産生が誘導されることを報告している(J Immunol. 2015)。悪性中皮腫患者の血清中 IL-10 濃度は健常者よりも顕著に高く、さらに中皮腫患者の中には極端に高値を示す患者もいたことは(図 1)、非常に興味深い結果である。悪性中皮腫細胞自身にも

CD26 のリガンドである caveolin-1 が発現しているため、中皮腫周囲に浸潤した T 細胞に CD26 共刺激が伝達して IL-10 の高産生を誘導している可能性が考えられる。ヒト化 CD26 抗体は CD26 に結合して、CD26 と caveolin-1 との結合阻害にも働くことから、免疫抑制性サイトカインである IL-10 の産生抑制に働くことが期待される。次年度では、国内第 I/II 相臨床試験被験者の血清検体を用いて、抗体投与前の血清中 IL-10 濃度および抗体投与による IL-10 濃度の変動に着目して、CD26 抗体の治療有効性との関係性を明らかにする。

近年、マウス担癌モデルにおいて、DPPIV inhibitor (Sitagliptin)をエサと一緒にマウスに食べさせることで、腫瘍免疫が増強し、腫瘍サイズが縮小することが報告された(Nat Immunol. 2015)。そのメカニズムとして、がん微小環境から産生されるケモカインの中で DPPIV 酵素の基質の一つである CXCL10 に着目し、本来は産生された CXCL10 が DPPIV 酵素による切断を受けると活性が低下するのに対し、DPPIV inhibitor を摂取することで、CXCL10 の活性が維持され、そのレセプターである CXCR3 陽性の CD4 T 細胞、CD8 T 細胞、NK 細胞ががん細胞周囲により集積するようになり、免疫細胞が腫瘍をより攻撃しやすくなることを示している。フランスでのヒト化 CD26 抗体の第 I 相臨床試験の結果から、CD26 抗体を投与すると容量依存的に血清中の可溶性 CD26 量および DPPIV 酵素活性が低下するデータを得ている(Br J Cancer. 2017)。このことから、CD26 抗体を投与した場合においても、DPPIV によって切断されるケモカインの量が減少し、ケモカインの活性維持、がん細胞周囲に集積する免疫細胞数の増加が起こる可能性が考えられる。悪性中皮腫患者の末梢血 T 細胞の CXCR3 の発現陽性率は CD4 T 細胞、CD8 T 細胞ともに、健常者と比較して低い傾向にあった(図 4)。しかしながら、図 4 に示したように、一部の中皮腫患者では CXCR3 の発現陽性率が高いことから、これらの CXCR3 の発現率が高い患者と CD26 抗体の治療有効性との関係性について今後着目したい。

がん微小環境に浸潤した T 細胞は、がん細胞自身が産生する免疫抑制性因子や PD1/PDL1・PDL2、CTLA4/CD80・CD86 に代表される免疫チェックポイント分子シグナルによって、エフェクター機能が抑制されていることが様々ながん種で報告されている。悪性中皮腫患者では末梢血 CD8 T 細胞中の細胞傷害性エフェクターT 細胞(CD28 陰性 CD57 陽性)の割合が健常者と比較して明らかに高く、さらに CD4 T 細胞においても細胞傷害活性を有するサブセットが存在することが示された(図 4)。このような perforin と granzyme を発現する細胞傷害性エフェクターT 細胞は本来、ウイルスに感染した細胞やがん細胞を体内から除去するために働く細胞だが、悪性中皮腫患者の末梢血中にどうして細胞傷害活性を有する T 細胞がこれほど多く存在するのか、何の抗原に対して活性化された T 細胞なのかは興味深い疑問である。免疫チェックポイント分子の中で、悪性中皮腫患者の末梢血 CD8 T 細胞は TIGIT の発現陽性率が健常者よりも明白に高く、また PD1 の発現も高い傾向が見られた(図 5)。免疫チェックポイント分子の発現は T 細胞の活性化状態や血清中の TGF-β 濃度などと関係していることが予想される。末梢血中の

細胞傷害性エフェクターT 細胞の割合や CD8 T 細胞の TIGIT 及び PD1 の発現と CD26 抗体の治療有効性との相関関係について次年度に解析を行う。

E. 結論

今年度は悪性中皮腫患者と健常者の血清中サイトカイン・ケモカインの多項目解析を行い、ヒト化 CD26 抗体の予後・治療効果予測バイオマーカー候補として、IL-10, MIF, Eotaxin/CCL11, IL-1β, IL-33 を見出した。また、国内第 I/II 相臨床試験患者の末梢血リンパ球のフェノタイプ解析を行い、悪性中皮腫患者では CXCR3 の発現が低下している傾向が見られ、また、特に CD8 T 細胞で CD26 陽性率の低下、細胞傷害性エフェクターT 細胞の増加、免疫チェックポイント分子の中で TIGIT と PD1 の発現陽性率の増加が認められた。

F. 今後の展望

次年度は、今年度に引き続き国内第 I/II 相臨床試験被験者や対照症例の解析検体数をさらに増やすとともに、血清中可溶性 CD26 濃度/DPPIV 酵素活性値に加え、上記のバイオマーカー候補の発現と、ヒト化 CD26 抗体の治療有効性との相関関係を解析し、予後・治療効果バイオマーカーを同定する。

G. 研究発表

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H. 知的財産権の出願・登録状況（予定を含む）

1. 特許取得
   発明者：大沼圭、森本幾夫、波多野良
   発明の名称:免疫チェックポイント阻害剤
   出願日：2019 年 1 月 15 日（PCT 出願）
   出願番号：特願 2019-04480
   出願人：学校法人順天堂
2. 実用新案登録
   なし
3. その他
   なし

厚生労働科学研究費補助金（労災疾病臨床研究事業）
分 担 研 究 報 告 書

悪性胸膜中皮腫におけるCD26発現評価とヒト化CD26抗体療法における予後・治療効果予測バイオマーカーの開発

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研究要旨
悪性胸膜中皮腫は現時点で効果的な治療法はなく、予後は極めて不良で労災疾病行政上も大きな問題であり、有効な新規治療法の確立は急務である。本邦で2017年7月から開始した悪性中皮腫の第I/II相臨床試験検体を用いて、病理組織でのCD26発現の定量・定性法の解析を行った。第I/II相臨床試験検体の中で、過去の病理組織の使用が許可された8症例について、病理組織像およびR&D社ポリクローナル抗体による免疫染色（フランスでの治験と同一の方法）によるCD26発現について検討した。その結果、組織型は上皮型が6例、ニ相型が1例、肉腫型が1例で、CD26発現は、上皮型では5%以下1例、50%2例、70%3例であり、二相型では、2%が1例、肉腫型では50%が1例であった。組織型およびCD26発現とヒト化CD26抗体の治療効果との関係に関しては、次年度により症例数を追加し、キッセイ社の臨床試験結果報告後に公開する。また、FFPE臨床検体におけるCD26発現評価が可能なモノクローナル抗体2クローン（U16-3, U38-8）を新規開発し、上記R&D社抗体との比較を行ったところ、U16-3, U38-8がR&D社抗体と同等以上のコンパニオン診断薬になりうるものと考えられた。

A. 研究目的

フランスで施行されたヒト化 CD26 抗体療法の第Ⅰ相臨床試験では、特記すべき有害事象もなく、26 症例中 13 症例で「安定」(Stable Disease;SD)への導入が可能であり、安全性のみならず、抗腫瘍効果も期待される成果が得られた(Br J Cancer 116:1126-1134, 2017)。本邦でも 2017 年 7 月から第Ⅰ相臨床試験がスタートし(第1～3 コホート各 3 例)、2018 年 3 月に最終被験者への投与が終了、フランスでの第Ⅰ相臨床試験と同様に、特記すべき有害事象もなく抗腫瘍効果も期待される成果が得られている(**具体的な結果に関しては、キッセイ社が臨床試験結果を報告した後に公開する**)。第 II 相臨床試験は 2018 年 6 月からスタートし、被験者のリクルートも非常に順調に進んでいる。そこで本研究においては、まず悪性中皮腫症例における CD26 発現を詳細に明らかにし、標的分子

としてどのような発現パターンを示すのかを明らかにし、CD26 抗体療法の治療効果との相関を明らかにするための基礎的検討を行った。また CD26 発現は現在用いられているアリムタ、シスプラチンなど化学療法剤の治療効果予測バイオマーカーとしても有望な結果を得て報告してきたが(Clin Cancer Res 18:1447, 2012)、さらに今回、CD26 発現を細胞局在、陽性率、陽性強度など様々な要素で解析し、各種の臨床パラメーターとの相関を明らかにすることで、バイオマーカーとなりうるかどうかを検討することを目的とした。

また、CD26 発現評価のためにホルマリン固定パラフィン包埋（FFPE）された臨床検体で CD26 を検出可能なモノクローナル抗体の新規開発を行った。

B. 研究方法

CD26 の発現解析には、ヒト組織としてホルマリン固定したパラフィン切片（CD26 陽性である正常ヒト腎、肝、前立腺及び悪性中皮腫の組織および肺）および生検や手術材料を用いた。抗原賦活化として、オートクレーブ処置（120℃、20 分、0.01M Citrate Buffer pH8.0）を行い、二次抗体は、Peroxidase 付加抗ラビット IgG 抗体(ImmPRESS 社製)を用い、発色は、DAB 液（Simple Stain DAB, Histofine）を用いた。岡山労災病院および山口宇部医療センターにおける中皮腫 84 症例の腫瘍の病理組織（生検及び手術材料、10%ホルマリン固定、パラフィン切片）について、CD26 の免疫染色を行った。抗原賦活化は、オートクレーブ処置（120℃、20 分、0.01M Citrate Buffer pH8.0)を行った。抗 CD26 抗体は、仏の臨床試験で使用した R&D 社製抗 CD26 ヤギ・ポリクロナール抗体(Lot.No. JOQ107061）を用いた。二次抗体は、Peroxidase 付加抗ヤギ IgG 抗体(ImmPRESS 社製)あるいは Peroxidase 付加抗マウス IgG 抗体(ImmPRESS 社製)を用い、発色は、DAB 液（Simple Stain DAB, Histofine）を用いた。いずれの染色においても、陽性対照には、正常ヒト腎、肝、前立腺及び悪性中皮腫を用い、陰性対照には、これらの正常組織切片内の各種組織（平滑筋、脂肪組織、結合組織など）と CD26 陰性肺癌組織を用いた。

新規モノクローナル抗体の作成は、可溶性 CD26 抗原を 8M 尿素処理にて変性させてからアジュバンドとともにマウスへ 8 回免疫した。常法通りに P3U1 骨髄腫細胞と融合させてからハイブリドーマを樹立した。上清の一次スクリーニングは、マウス IgG の ELISA にて行い、マウス IgG 高産生クローンは全て二次スクリーニングを行った。二次スクリーニングは、CD26 陽性および陰性細胞をサイトスピンにて添付したスライドグラスをホルマリン固定したものを用いて、免疫染色と同様の手法にて行った。さらに三次スクリーニングとして FFPE 切片(CD26 陽性である正常ヒト腎、肝、前立腺及び悪性中皮腫の組織および肺）の免疫染色を行った。

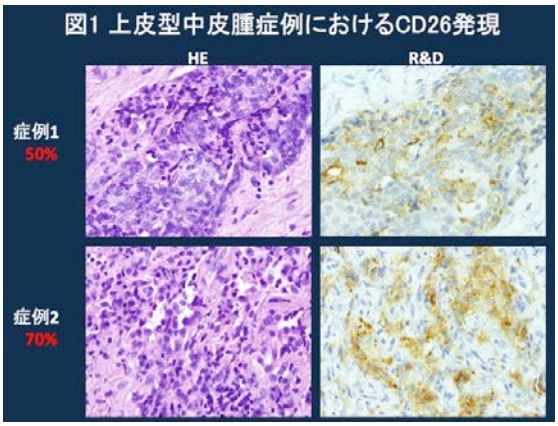
（倫理面への配慮）

患者検体などについては研究対象者に対する人権擁護上の配慮及び研究により研究対象者が受ける不利益、利益等の説明を患者及び遺族に対して行い、書面でのインフォームド・コンセントを得ている。また病理組織について免疫染色して CD26 発現を解析する研究については、埼玉医科大学の倫理委員会の審査にて承認されている（承認番号 794, 861）。

C. 研究結果

国内第 I 相臨床試験における悪性中皮腫 9 症例および第 II 相臨床試験における悪性中皮腫 3 症例の中で、過去の病理組織の使用が許可された 8 症例について、病理

組織像および R&D 社ポリクローナル抗体による免疫染色(フランスでの治験と同一の方法)による CD26 発現について検討した。8 症例は、上皮型中皮腫 6 例、二相型中皮腫 2 例(1 例は生検ではほとんど肉腫型)であり、CD26 発現は、上皮型で、5%以下 1 例、50%2 例、70%3 例であり、二相型では、2%が 1 例、肉腫型では 50%が 1 例であった(図 1)。組織型および CD26 発現とヒト化 CD26 抗体の治療効果との関係については、現時点では症例数が少ないため、次年度により症例数を追加して、第 II 相臨床試験が終了した時点で解析を行う予定である。



これらの染色標本をデジタル画像とし、imageProPlus プログラムにて詳細に半定量的に発現解析を行った。その結果、CD26 は細胞膜および細胞質に 95%が、核内に 1-2%の発現が認められた。現在、これらのデータベースを元に臨床パラメーターとの相関について検討している。

また FFPE 臨床検体における CD26 発現評価が可能な新規開発したモノクローナル抗体 2 クローン(U16-3, U38-8)と上記 R&D 社ポリクローナル抗体との比較を行った(図 2)。その結果、中皮腫 69 症例において、抗体 3 種類での陽性一致率は、94%であり、陽性率 10%以上での不一致例は 0 例であった(図 3)。また抗体 3 種類における陽性パターン(細胞膜、細胞質)は、ほぼ完全に一致していたが、陽性例における S/N 比は、U38-8 がもっとも優れていた。この結果は、新規モノクローナル抗体 U16-3, U38-8 は、R&D 社ポリクローナル抗体と同等以上のコンパニオン診断薬になりうるものと考えられた。

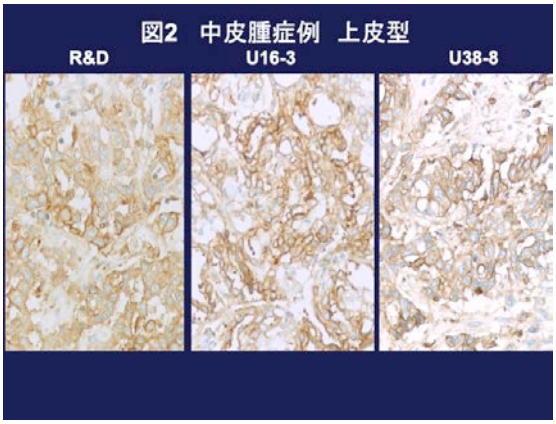


図3 U16-3, U38-8, R&Dポリクローナル抗体によるCD26発現

	陽性率 10%≦	陽性率 20%≦	細胞膜 >細胞質	細胞膜 <細胞質
U16-3	63/69 91%	57/69 83%	35/69 51%	7/69 10%
U38-8	63/69 91%	60/69 87%	32/69 46%	9/69 13%
R&D	63/69 91%	59/69 86%	35/69 49%	5/69 7%
一致率	100%	94%		

D. 考察

悪性中皮腫における CD26 発現について免疫染色にて評価し、組織型とともに発現パターン(細胞局在、陽性率、陽性強度などの各種パラメーター)を詳細に定量評価することにより、抗体療法の効果や予後などとの関連性あるいは臨床パラメーターを検証する基礎を構築していくことが重要と考える。

E. 結論

臨床試験における悪性中皮腫検体における CD26 発現評価を行い、半定量的解析法を確立した。また FFPE 検体で使用可能な新規モノクローナル抗体 U16-3, U38-8 の開発を通じて、CD26 抗体療法におけるコンパニオン診断薬に有用であることを見出した。

F. 今後の展望

今年度と継続して国内第ＩＩ相臨床試験の悪性中皮腫の病理組織像および CD26 発現を解析し、組織型や CD26 発現の細胞局在、陽性率、陽性強度、細胞増殖期率など中皮腫の様々な病理学的パラメーターを解析し、ヒト化 CD26 抗体の有効性評価項目との相関を統計学的に解析することで、CD26 抗体の予後・治療効果予測バイオマーカーとなりうるかどうかを検討する。

G. 研究発表

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4) 松永洸昂、三井絵里花、金 玲、山田健人、佐々木惇 DICを呈した骨髄増殖性腫瘍の1剖検例
第107回日本病理学会総会 札幌・ロイトン札幌 2018年6月21日(木)～23日(土)

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H. 知的財産権の出願・登録状況（予定を含む）

1. 特許取得

名称 抗ヒトCD26モノクローナル抗体
出願人（権利者） 学校法人順天堂、学校法人埼玉医科大学（共同出願）
発明人 森本幾夫、波多野良、山田健人、大沼圭.
出願日 2018年 3月16日(PCT出願)
出願番号 特願2018-049308

2. 実用新案登録

なし

3. その他

なし

労災疾病臨床研究事業費補助金

分担研究報告書

―脱ユビキチン化酵素 USP22 は新しい悪性中皮腫治療ターゲット―

予後および CD26 抗体治療効果予測のバイオマーカーになり得るか？

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研究要旨

悪性胸膜中皮腫は効果的な治療法が存在しないため、新しい治療法の開発が待たれている。我々は CD26 が中皮腫に発現していて、増殖・浸潤に関与していることを明らかにしてきた。さらに、ヒト化 CD26 モノクローナル抗体の開発に成功し、本抗体が標準化学療法抵抗性の中皮腫患者にも効果を示す結果を得ている。一方、USP22 は新規の脱ユビキチン化酵素として発見された核蛋白である。がん細胞内では USP22 はヒストンの脱ユビキチン化を通じて、特定の遺伝子の活性化や細胞周期の調節に関与している。種々のがんで USP22 が過剰に発現していること、進行度や予後に相関していることがわかってきたが、中皮腫における USP の発現や機能については報告がなされていない。

そこで本研究では、p21 を介した CD26 による細胞周期調節と関連し、中皮腫の増殖・浸潤における USP22 の役割を調べた。その結果、USP22 蛋白は中皮腫で発現し、USP22 ノックダウンは CD26 ノックダウンと同様に、中皮腫の増殖を阻害すること、両者を同時にノックダウンすると、その効果がさらに増強することを明らかにした。USP22 と CD26 の発現は相関し、ヒト化 CD26 抗体処理は CD26 を通じて USP22 の発現レベルを下げ、結果的にヒストン H2A と p21 のユビキチン化を増加させた。これらの結果から、USP22 は中皮腫治療における新しい標的になりうるとともに、予後予測バイオマーカーとなる可能性も示唆され、今後、悪性中皮腫の USP22 発現と予後や CD26 抗体の治療反応性との関係性についても評価したい。

A. 研究目的

悪性胸膜中皮腫は現時点で効果的な治療法はなく、予後は極めて不良で労災疾病行政上も大きな問題であり、有効な新規治療法の確立は急務である。

研究代表者は CD26 単クローン抗体の開発、CD26 cDNA の単離を世界に先駆けて行い(J Immunol. 1989, 1992)、抗腫瘍効果の強いヒト化 CD26 抗体の開発に成功した。悪性中皮腫における CD26 発現の解析、抗体の抗腫瘍作用機構の解明に取り組み、この抗体は抗体医薬特有の ADCC に加え、CD26 陽性腫瘍に結合することで p21 などの cyclic dependent kinase inhibitor(CDKI)を誘導して細胞周期を停止させて腫瘍の増殖抑制に働くこと、さらに近年では腫瘍免疫の促進にも働きうることを明らかにしてきた(Clin Cancer Res. 2007, 2012, PLoS One. 2013, Br J Cancer. 2014, Nat Immunol. 2015, Cancer Cell Int. 2016)。さらに、抗体療法の確立に不可欠な病理組織の CD26 発現診断用抗体、可溶性 CD26/DPPIV 値測定系を開発し、フランスにて治療抵抗性悪性中皮腫を中心とした First-in-Human 第 I 相臨床試験を施行した。その結果、**免疫チェックポイント阻害薬のような特記すべき有害事象もなく、**さらに**有効性を示唆するデータも得られた**(Br J Cancer. 2017)。

上述のように CD26 抗体の抗腫瘍作用メカニズムの一つに細胞周期の停止があるが、がん細胞の細胞膜上の CD26 分子に CD26 抗体が結合することで何故細胞周期の停止が生じるのかについては、詳しい作用機序は不明であった。脱ユビキチン酵素の USP22 は SAGA complex 転写因子としても知られているが、様々ながんで発現しており、高発現していると予後が悪いこと、stage が進行したがんで高発現していることなどから、予後因子としても有用であると考えられている。さらに、USP22 はヒストンの脱ユビキチン化を通じて、特定の遺伝子の活性化や細胞周期の調節にも関与しているといわれている。しかしながら、悪性中皮腫細胞における USP22 の発現や機能は不明である。

そこで本研究では、悪性中皮腫における CD26 分子と USP22 を介した細胞周期の関係および CD26 抗体処理による影響について解析を行い、USP22 が新たな治療ターゲットになり得るか、また CD26 抗体治療における USP22 の意義について検討することを目的とした。

B. 研究方法

1) 細胞と抗体

中皮腫細胞株 MESO1 は 理研バイオリソースセンター、JMN 株は Brenda Gerwin 博士(Laboratory of Human Carcinogenesis, NIH)からの寄贈、ヒト化 CD26 モノクローナル抗体は Y's AC 社から入手した。

2) 組織標本と免疫組織化学

患者由来中皮腫検体は慶応大学医学部の規則に則り適切に処理した。免疫組織化学の方法は、過去の我々の文献を参照のこと。

3) フィローサイトメトリー解析と免疫染色

細胞は固定・膜透過し、抗体と反応させたのち、FACS Calibur(BD)で解析した。細胞分離には FACSAria を用いた。データは FACS Diva と FlowJo ソフトウエアで解析した。免疫細胞化学は過去に記述した我々の方法を用いた。

4) cDNA 遺伝子導入

中皮腫細胞に shRNA を遺伝子導入するには、レンチウイルスシステムを用いた。USP22 の全長 cDNA を導入するには pDON5ベクターと Lipofectamine を用いた。

5) 免疫沈降とウエスタンブロット

細胞懸濁液をヒト化 CD26 モノクロ抗体と混ぜ、Protein G で吸着させたものを洗浄し、通常の方法でウエスタンブロットを行った。定量は C-Digit Blot Scanner を用いた。

6) 細胞増殖アッセイとマウス移植実験

細胞増殖は MTT アッセイを過去のプロトコール通りに行った。マウス移植実験は背部皮下または経静脈的に中皮腫細胞を注射し、経時的に生存を計測した。

7) 統計

データは Student T 検定を行い、統計的に処理した。生存曲線は Kaplan-Meier 解析で計算した。

（倫理面への配慮）

ヒト化 CD26 抗体、CD26 および USP22 ノックダウンの抗腫瘍効果を評価するためのマウスを用いた動物実験は、順天堂大学医学部実験動物委員会の審査を受け承認されている（承認番号 270138）。

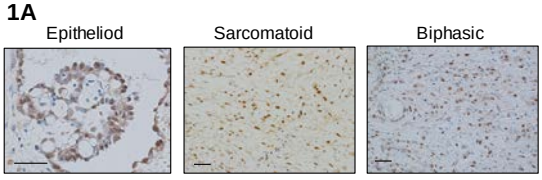
患者検体などについては研究対象者に対する人権擁護上の配慮及び研究により研究対象者が受ける不利益、利益等の説明を患者及び遺族に対して行い、書面でのインフォームド・コンセントを得ている。また病理組織について免疫染色して CD26 発現を解析する研究については、慶應義塾大学医学部及び埼玉医科大学の倫理委員会の審査にて承認されている（承認番号 20120100 及び 734）。

C. 研究結果 および D. 考察

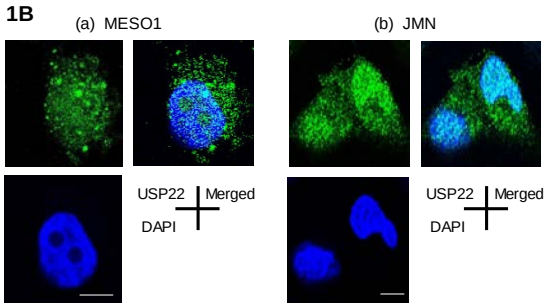
1) 中皮腫臨床サンプルにおける USP22 の発現と、USP22 ノックダウンの細胞増殖阻害効果

USP22 はさまざまながんで発現が報告されているが、悪性中皮腫ではまだ詳しくは調べられておらず、我々はまずそれについて実験を行った。その結果、USP22 は悪性中皮腫の全ての組織型（上皮型、肉腫型、混合型）で発現がみられた。上皮型では 26 例中 21 例(81%)で陽性だったのに対し、肉腫型では 5 例中 3 例(60%)、混合型では 12 例中 7 例(58%)が陽性だった。図 1A はその代表的な写真を示している。

次に我々は、中皮腫細胞株でも USP22 の発現を調べたが、図 1B で示すように、MESO1 株と JMN 株では核と細胞質の両方で USP22 の発現が見られた。



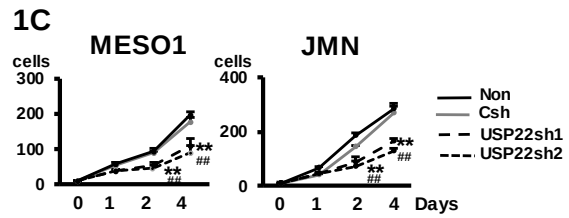
1A) 中皮腫病理検体（上皮型・肉腫型・混合型）における代表的な USP22 の免疫組織化学写真。USP22 はそれぞれの組織型で高発現している。（スケールバー：50μm）



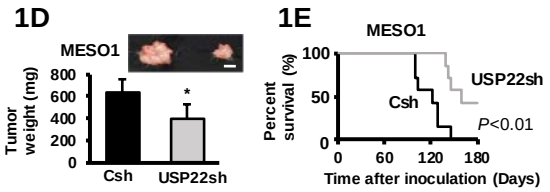
1B) 中皮腫細胞株（MESO1）における USP22 の共焦点顕微鏡写真。USP22 は細胞質と核の両方に発現しているが、細胞表面にはほとんど見られない。（スケールバー：10μm）

USP22 の shRNA ノックダウン実験を行ったところ、その増殖が抑制された。しかも

その細胞は、マウス移植実験でも腫瘍増殖が抑制され、生存も延長した(図 1C-E)。これらの結果からUSP22のノックダウンは中皮腫の増殖と成長を抑制することが示された。



1C) USP22-shRNA-1, -2 を細胞株(MESO1 とJMN)に遺伝子導入したところ、ともに細胞増殖は有意に減速した。



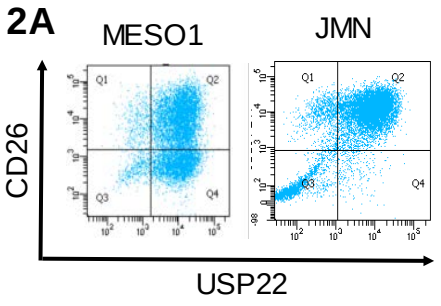
1D) USP22-shRNA とコントロール shRNA を安定発現する細胞を作成し、SCID マウスの背部皮下に移植して、10 日目に腫瘍の重さを測定した。すると、USP22-shRNA の方で有意に腫瘍形成が抑制された。上段に代表的な顕微鏡写真を示す。(スケールバー:1μm)

1E) 上記の細胞を静脈注射し、生存をカプラン・マイヤー解析で評価したところ、USP22-shRNA の方で有意に生存が延長した。

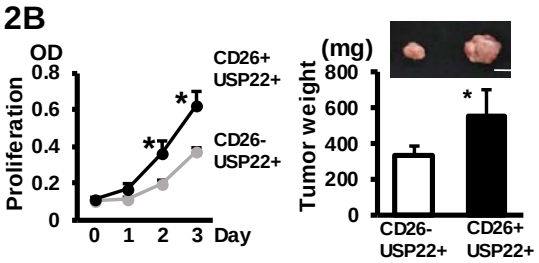
2) USP22 と CD26 のダブルノックダウンは、いずれか一方のノックダウンより、中皮腫細胞の増殖を強く阻害する

我々は以前、CD26 分子のノックダウンが中皮腫の増殖と浸潤を抑制することを報告している。また、CD26 は中皮腫においてもがん幹細胞特性と相関し、がん幹細胞マーカーであることを示した。一方、我々は以前の報告で、USP22 が B-ALL を始め、さまざまながんでがん幹細胞特性に関連していることを発見した。そのため、USP22 と CD26 が協調効果を示すかどうかを調べた。MESO1 株は CD26+USP22+ と CD26-USP22+ 分画を含んでいるのに対し、JMN 株はほとんど CD26+USP22+ 分画細胞だけから成っていた(図 2A)。

次に MESO1 株を CD26+と CD26-に分離して解析したところ、図 2B のように、CD26+細胞の方が CD26- 細胞よりも in vitro で培養した時の細胞増殖および in vivo でマウスに皮下移植した時の増殖が速かった。これからの結果から、CD26 は USP22 と協調して増殖を促進していることが示唆された。



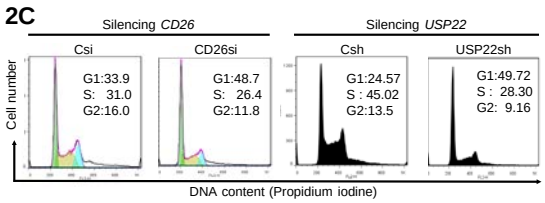
2A) 代表的な CD26(PE)と USP22(FITC)の二次元プロット。MESO1 細胞は CD26+USP22+と CD26-USP22+分画を含む。一方、JMN は主に CD26+USP22+とわずかな CD26+USP22-分画を含む。



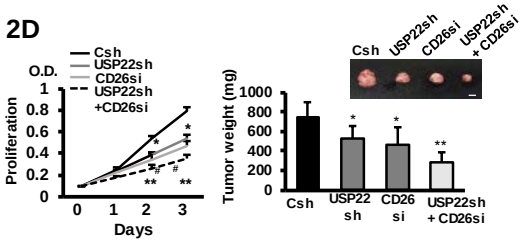
2B) MESO1 の CD26+または CD26-細胞をフローサイトで分離したところ、いずれも USP22 が発現しているのが観察された。MTT アッセイで CD26+USP22+と CD26-USP22+細胞分画の増殖を測定したところ、CD26+USP22+の方が高い増殖活性を示した。SCID マウス皮下移植実験でも、CD26+USP22+細胞が高い造腫瘍活性を示した。

我々は以前、CD26 発現減少が CDKI の

p21 発現増強を介した細胞周期調節に関わっていることを報告し、USP22 も CDKI の p21 を標的にしていることを明らかにした。そこで CD26 と USP22 ノックダウン細胞の細胞周期解析を行ったところ、G1/S arrest がおこっていたことが判明した(図 2C)。これらのことから、CD26 と USP22 は細胞周期に影響することで、阻害効果を示すことがわかった。また両者のノックダウンでは、その効果が増強された(図 2D)。



2C) CD26-siRNA または USP22-shRNA を導入した MESO1 の細胞周期解析では、コントロールに比較して G1 期の増加および、S 期と G2/M 期の減少が観察された。

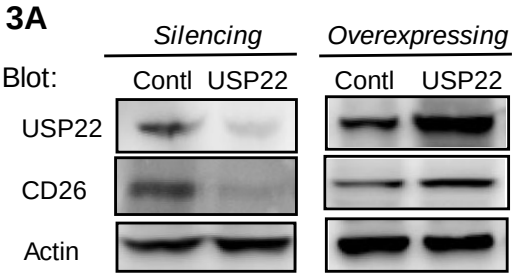


2D) MTT アッセイで、USP22-shRNA, CD26-siRNA, その両者同時、およびコントロールを遺伝子導入した細胞の増殖を調べたところ、単独よりも両者同時のノックダウンで増殖が最も抑制された。それらを SCID マウス移植実験に用いると、同様に両者同時が最も腫瘍形成が抑制された。

悪性中皮腫患者検体において、CD26 陽性の中皮腫のうち USP22 も発現している症例は上皮型で 71%、肉腫型で 33%、混合型で 71%だったが、CD26 と USP22 が共陽性の中皮腫患者の方が予後不良である傾向が見られており、CD26 と USP22 が協調して中皮腫の悪性化に関与していることが示唆された。

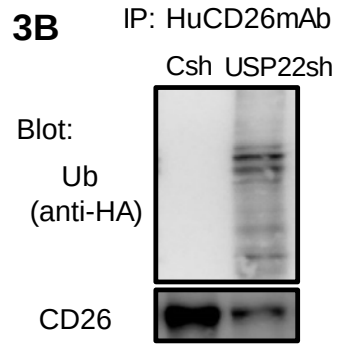
3) ヒト化 CD26 抗体は CD26 と USP22 を介して中皮腫細胞の増殖を抑制する

上記の実験で、CD26 と USP22 に相互作用が示唆されたため、それぞれの発現がお互いの発現に影響するかどうかを調べた。その結果、USP22 をノックダウンすると CD26 発現も減少し、USP22 を過剰発現させると CD26 発現も増加することが示された(図 3A)。



3A) USP22-shRMNA を安定発現させた MESO1 細胞における、USP22 と CD26 のウエスタンブロット解析。USP22 ノックダウンにより CD26 が減少し、USP22 を過剰発現させると CD26 も増加する。

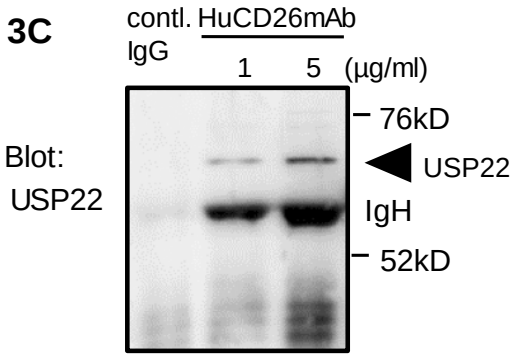
USP22 は脱ユビキチン化酵素活性を持つため、次に USP22 発現に関連した CD26 のユビキチン化状態を調べた。その結果、USP22 の発現が減少すれば CD26 のユビキチン化が増加していることが示された(図 3B)。このことから、USP22+CD26+中皮腫細胞では、USP22 が CD26 の発現を調節していることが示唆された。



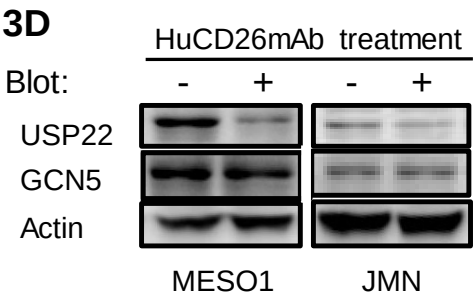
3B) USP22-shRNA 安定発現細胞を HA-tag 付きユビキチンと培養した後、細胞懸濁液を HuCD26mAb で免疫沈降したサンプルを、抗 HA 抗体と CD26 抗体で検出した。抗体処理の結果、CD26 蛋白は減少したが、沈降物はコントロール細胞に比較して、USP22-shRNA 細胞の方で有意にユビキチン化が増加していた。

CD26 は主に細胞表面に発現しているのに対して、USP22 は SAGA 転写因子コファクター複合体の一つであり核に局在している。我々は最近、細胞表面上の CD26 分子にヒト化 CD26 抗体が結合すると、CD26 とヒト化 CD26 抗体が複合体を形成したまま細胞内、さらには核に移行し、中皮腫細胞の増殖抑制に作用することを示したが、この核に移行した CD26 が USP22 に作用し、USP22 の発現減少と p21 の発現上昇を引き起こすと推測した。

図 3C で示すように、ヒト化 CD26 抗体の用量依存的に CD26 と USP22 は複合体を形成し、しかもそれは、核内 USP22 量の減少をもたらしたが、別の SAGA 複合体である GCN5 には影響しなかった(図 3D)。



3C) HuCD26mAb 処理後の細胞(MESO1)の免疫沈降で、USP22 が HuCD26mAb により共沈されてきた。



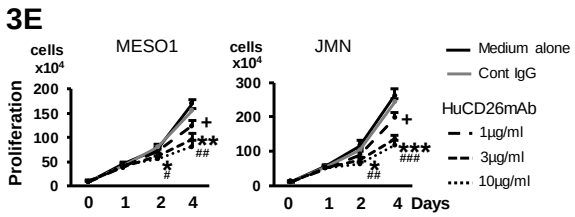
3D) HuCD26mAb 処理した細胞の核抽出液のウエスタンブロット解析。HuCD26mAb 処理で USP22 の発現は抑制されるが、GCN5 の発現は抑制されなかった。

USP22 の鍵となる機能は、p21 発現を調節するヒストン H2A の脱ユビキチン化だが、我々はさらにこの点を検討した。その結果、ヒト化 CD26 抗体処理はヒストン H2A の脱ユビキチン化と p21 の発現レベルを増強させることがわかった(表 1)。同様の結果は USP22 のノックダウンでも得られた。しかもヒト化 CD26 抗体処理は、用量依存的に細胞増殖を阻害し(図 3E)、G1/S arrest を引き起こした(図 3F)。

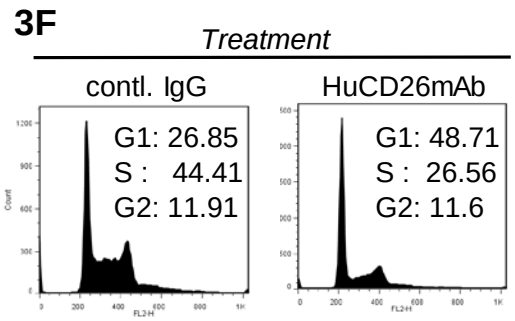
表 1

	Ubiquitinated histone H2A(%)	p21(%)
Control-shRNA	95.6	32.5
USP22-shRNA	98.4*	40.8*
Control IgG	93.3	64.0
HuCD26mAb	98.8**	68.2**

表 1）USP22-shRNA-1 安定導入または HuCD26mAb 処理 MESO1 細胞を抗ユビキチン化ヒストン H2A 抗体または抗 p21 抗体処理し、FITC 二次抗体で染めてからフローサイト解析を行った。コントロールに比較し、両細胞ともにユビキチン化ヒストン H2A と p21 が有意に増加していた。

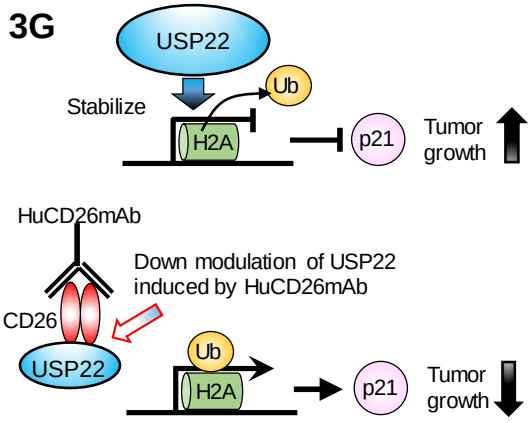


3E）HuCD26mAb 処理細胞を MTT アッセイで解析したところ、細胞増殖は抗体濃度に依存して抑制された。



3F）HuCD26mAb 処理細胞の細胞周期解析では G1 期が増え、S 期と G2/M 期が減少した。

これらの結果は、ヒト化 CD26 抗体処理が核内の USP22 を減少させることで、中皮腫細胞の増殖を抑制することを強く示唆している。実験結果より、USP22 による細胞周期調節と中皮腫細胞の増殖における、ヒト化 CD26 抗体の影響のモデルを図 3G に示す。すなわち、恒常的な USP22 の高発現はヒストン H2A の脱ユビキチン化を安定化し、ヘテロクロマチンをサイレンシングすることで p21 発現を抑制し、その結果、腫瘍の異常増殖を引き起こす。一方、ヒト化 CD26 抗体処理による CD26 の核移行は核内での CD26-USP22 複合体形成を引き起こし、核内の USP22 量を減少させる。これにより、ヘテロクロマチンサイレンシングが解除され、CDKI p21 を含む特定の遺伝子を活性化し、腫瘍の増殖を抑制すると考えられる。



今回の研究により CD26 抗体処理による細胞周期停止および CDKI p21 の発現誘導の分子メカニズムを明らかにでき、USP22 は新たな治療ターゲットになり得、また USP22 の悪性中皮腫での発現評価はヒト化 CD26 抗体の治療反応性予測などのバイオマーカーとなる可能性も示唆された。

E. 結論

USP22 の発現抑制は中皮腫細胞の増殖を抑制した。ヒト化 CD26 抗体処理は細胞膜上の CD26 を核移行させ、UPS22 と CD26 を核内で物理的に接触させることにより、CDKI p21 の発現上昇と腫瘍の増殖抑制を引き起こすことを明らかにした。USP22 は様々ながんの治療標的となり得るが、核内蛋白なので抗体の直接の標的にするのは困難

である。その一方で、ヒト化 CD26 抗体による CD26 標的化は USP22 を減少させることが可能なため、将来的には中皮腫のみならずさまざまな CD26 陽性がんで、有用なアプローチになり得、またその悪性中皮腫での発現評価はヒト化 CD26 抗体の治療反応予測となる可能性を示唆された。

F. 今後の展望

悪性中皮腫組織細胞における USP22 の発現評価は悪性中皮腫における予後およびヒト化 CD26 抗体の治療反応性予測のバイオマーカーとなり得るかどうかについても今後解析を行う予定である。

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月 26 日、東京（東京国際フォーラム）

H. 知的財産権の出願・登録状況（予定を含む）

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Ⅲ.　研究成果の刊行に関する一覧表

＜研究成果の刊行に関する一覧表＞

【書　籍】

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
Hatano R, Ohnuma K, Yamada T, Okamoto T, Komiya E, Otsuka H, Itoh T, Yamazaki H, Iwao N, Kaneko Y, Dang NH, Morimoto C	The use of the humanized anti-CD26 monoclonal antibody YS110 as a novel targeted therapy for refractory cancers and immune disorders	Leon V. Berhardt	Advances in Medicine and Biology	Nova Science Publishers,Inc	USA	2018年	1-44
岸本卓巳	石綿関連疾患研究の現在. 産業医学のプリンシプル～大切なこと	「産業医学ジャーナル」編集委員会	産業医学振興財団40周年記念誌	公益財団法人産業医学振興財団	東京	2018年	247-50

【雑　誌】

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Ⅳ.　研究成果の別刷

Chapter

THE USE OF THE HUMANIZED ANTI-CD26 MONOCLONAL ANTIBODY YS110 AS A NOVEL TARGETED THERAPY FOR REFRACTORY CANCERS AND IMMUNE DISORDERS

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ABSTRACT

CD26 is a 110 kDa surface glycoprotein with intrinsic dipeptidyl peptidase IV (DPPIV) activity that is expressed on numerous cell types and has a multitude of biological functions. We have had a long-standing interest in the role of CD26 in cancer biology and immune regulation and developed YS110, a humanized monoclonal antibody (mAb) with high affinity to the CD26 antigen. Our group has shown that *in vivo* administration of YS110 inhibits tumor cell growth, migration and invasion, and enhances survival of mouse xenograft models inoculated with malignant mesothelioma (MPM), renal cell carcinoma, non-small-cell lung carcinoma, ovarian carcinoma or T-cell lymphoma via multiple mechanisms of action. The first-in-human (FIH) phase 1 clinical trial of this mAb for CD26-expressing solid tumors, particularly refractory MPM, was conducted from 2009 to 2014, with results being recently published. Our FIH study demonstrates that YS110 therapy exhibits a favorable safety profile and results in encouraging disease stabilization in a number of patients with advanced/refractory CD26-expressing cancers. In addition, we have robust evidence through multiple studies that CD26 functions as a T-cell costimulatory molecule and has an important role in T-cell biology. High CD26 cell surface expression is correlated with enhanced migratory ability through endothelial cells, and $CD26^+$ T cells are present at high levels in the inflammatory site of graft-versus-host disease (GVHD) and various autoimmune diseases such as rheumatoid arthritis, multiple sclerosis and Graves' disease. Our recent work with a xenogeneic GVHD murine model also demonstrates that administration of YS110 decreases GVHD severity and prolongs survival, while preserving the graft-versus-leukemia effect. Furthermore, recent work has shown that infection by the novel Middle East respiratory syndrome coronavirus (MERS-CoV) is mediated by the use of CD26 as a functional receptor. We have identified the domains of CD26 involved in the binding of MERS-CoV and showed that YS110 treatment significantly inhibits viral infection. We herein review novel findings strongly suggesting that YS110 represents a promising novel therapy for refractory cancers, immune disorders and MERS-CoV infection.

Keywords: CD26/DPPIV, YS110, malignant mesothelioma, immune disorders, MERS-CoV

1. INTRODUCTION

CD26 is a 110-kDa, type II transmembrane glycoprotein with known dipeptidyl peptidase IV (DPPIV, EC 3.4.14.5) activity in its extracellular domain and is capable of cleaving N-terminal dipeptides with either L-proline or L-alanine at the penultimate position (Ohnuma et al., 2008a). CD26 activity is dependent on cell type and the microenvironment factors that can influence its multiple biological roles (Ohnuma et al., 2011; Thompson et al., 2007). CD26 is expressed on various tumors including malignant pleural mesothelioma (MPM), renal cell carcinoma (RCC), lung cancer, colorectal cancer (CRC), hepatocellular carcinoma, prostate cancer, gastrointestinal stromal tumor (GIST), thyroid carcinoma, and hematologic malignancies such as T-anaplastic large cell lymphoma, T-lymphoblastic lymphoma and T-acute lymphoblastic leukemia (Havre et al., 2008; Ohnuma and Morimoto, 2013; Thompson et al., 2007). We have had a long-standing interest in the role of CD26 in cancer biology and developed YS110, a humanized monoclonal antibody (mAb) with high affinity to the CD26 antigen. Our group has shown that *in vitro* and *in vivo* administration of YS110 inhibited tumor cell growth, migration and invasion, and enhanced survival of mouse xenograft models inoculated with MPM, RCC, non-small-cell lung carcinoma, ovarian carcinoma or T-cell lymphoma via multiple mechanisms of action (Inamoto et al., 2007; Inamoto et al., 2006; Ho et al., 2001). The first-in-human (FIH) phase 1 clinical trial of this mAb for CD26-expressing solid tumors, particularly refractory MPM, was conducted with results being recently published (Angevin et al., 2017). Our FIH study demonstrated that YS110 therapy exhibits a favorable safety profile and resulted in encouraging disease stabilization in a number of patients with advanced/refractory CD26-expressing cancers.

In addition, we have robust evidence through multiple studies that CD26 functions as a T-cell costimulatory molecule and has an important role in T-cell biology and overall immune function (Morimoto and Schlossman, 1998; Ohnuma et al., 2008a). We identified caveolin-1 as a costimulatory ligand for CD26 in T cells, and showed that CD26-caveolin-1 interaction led to activation of both CD4 T cells and antigen presenting cells (APCs) (Ohnuma et al., 2004; Ohnuma et al., 2005; Ohnuma et al., 2007). Moreover, High CD26 cell surface expression was correlated with enhanced migratory ability through endothelial cells (Masuyama et al., 1992), and $CD26^+$ T cells were present at high levels in the inflammatory site of graft-versus-host disease (GVHD) and various autoimmune diseases such as rheumatoid arthritis (RA), multiple sclerosis and Graves' disease (Hatano et al., 2013a; Ohnuma et al., 2015a; Ohnuma et al., 2011). Our recent work with a xenogeneic GVHD murine model also demonstrated that administration of YS110 decreased GVHD severity and prolonged survival, while preserving the graft-versus-leukemia (GVL) effect (Hatano et al., 2013a).

Furthermore, recent work has shown that infection by the Middle East respiratory syndrome coronavirus (MERS-CoV) is mediated by the use of CD26 as a functional receptor (Raj et al., 2013). MERS-CoV is a novel coronavirus identified in patients with severe lower respiratory tract infections with almost 50% of cases resulting in lethal lower respiratory tract infections (Zaki et al., 2012; Enserink, 2013). We have identified the domains of CD26 involved in the binding of MERS-CoV and showed that YS110 treatment significantly inhibited viral infection (Ohnuma et al., 2013). We recently reviewed our significant findings and the early clinical development of a CD26-targeted therapy for MPM (Ohnuma et al., 2017). We herein review novel findings strongly suggesting that YS110 represents a promising novel therapy not only for MPM but also for other refractory cancers, immune disorders and MERS-CoV infection.

2. CANCERS

2.1. Malignant Pleural Mesothelioma

Our recent in-depth studies of CD26 expression in MPM revealed that CD26 is preferentially expressed in MPM cells but not in normal mesothelial cells (Amatya et al., 2011; Aoe et al., 2012). Our immunohistochemical analysis showed that membranous expression of CD26 was particularly found in epithelioid mesotheliomas, but not in sarcomatoid mesotheliomas (Aoe et al., 2012). In addition, we identified SP (side-population), CD9, CD24, and CD26 as MPM cancer stem cell markers that correlated with primary stem cell signatures (Ghani et al., 2011; Yamazaki et al., 2012). These intriguing findings propelled our development of CD26-targeted therapy for MPM. For this purpose, we had developed a novel humanized anti-CD26 mAb, YS110. We recently reviewed the functional role of CD26 in the neoplastic biology of MPM and our robust *in vitro* and *in vivo* data investigating the anti-tumor effect of YS110 on MPM (Ohnuma et al., 2017). We conducted the FIH phase 1 clinical trial of YS110 for patients with refractory MPM and other CD26-positive solid tumors (Angevin et al., 2017). Thirty-three heavily pre-treated patients with CD26-positive cancers including 22 MPM underwent YS110 administration. Our FIH study conclusively demonstrated that YS110 exhibits a favorable safety profile and substantial clinical activity in heavily pre-treated CD26-positive MPM patients who had previously progressed on conventional standard chemotherapies. Further clinical trial of YS110 for MPM is in progress worldwide (ClinicalTrials.gov, 2017).

2.2. Other Cancers

In contrast to our robust findings regarding the role of CD26/DPPIV on MPM (Ohnuma et al., 2017), the exact role of CD26/DPPIV in other cancers remains to be elucidated, partly due to its variable expression on these tumors. In general, it is strongly expressed on some cancers, while

being absent or present at low levels in others (Thompson et al., 2007). Furthermore, given its multiple biological functions, including its ability to associate with several key proteins and its cleavage of a number of soluble factors to regulate their function, it is likely that the CD26/DPPIV effect on tumor biology is at least partly mediated by the effect of these biological functions on specific tumor types (Havre et al., 2008).

2.2.1. Renal Cancer

CD26 has also been shown to be expressed on RCC (Stange et al., 2000; Inamoto et al., 2006; Hatano et al., 2014), including the cell lines Caki-1, Caki-2, VMRC-RCW, and ACHN (Inamoto et al., 2006). We showed that anti-CD26 mAb inhibition of the Caki-2 cell line was associated with G1/S cell cycle arrest, enhanced $p27^{kip1}$ expression, downregulation of cyclin-dependent kinase 2 (CDK2) and dephosphorylation of retinoblastoma substrate (Rb) (Inamoto et al., 2006). We also found that anti-CD26 mAb therapy attenuated Akt activity (Figure 1D) and internalized cell surface CD26 leading to decreased CD26 binding to collagen and fibronectin (Figure 1A and 1B). Treatment with anti-CD26 murine mAb inhibited the growth of human RCC and significantly enhanced survival in a mouse xenograft model (Figure 1C). Our FIH phase 1 study demonstrated that disease stabilization was observed in heavily pre-treated CD26-positive MPM and RCC patients who had previously progressed on conventional standard therapies (Angevin et al., 2017), suggesting that YS110 treatment may have potential clinical use for CD26-positive RCC.

2.2.2. Lung Cancer

CD26 expression in lung cancer appears to be dependent on the specific histologic subtype. Liu et al., showed that CD26 was highly expressed in poorly differentiated lung adenocarcinomas compared to highly differentiated lung adenocarcinomas utilizing human lung adenocarcinoma tissue microarrays (Liu et al., 2013). These investigators demonstrated that CD26 inhibition by shRNA significantly decreased the invasive and migratory capacity of human lung adenocarcinoma cell line,

SPC-A-1sci cells, while CD26 overexpression increased the invasive and migratory capacity of SPC-A-1 cells (Liu et al., 2013). We are currently investigating *in vitro* and *in vivo* anti-tumor activity of YS110 on lung cancers, and our preliminary data show that treatment with YS110 inhibits the growth of human lung cancer cell lines *in vitro* and *in vivo*. Definitive data will be presented in the near future.

2.2.3. Colorectal Cancer

Pang et al., identified a subpopulation of $CD26^+$ cells uniformly presenting in both primary and metastatic tumors in colorectal cancer, and showed that $CD26^+$ cancer cells are associated with enhanced invasiveness and chemoresistance (Pang et al., 2010). These investigators showed that in $CD26^+$ colorectal cancer cells, mediators of epithelial to mesenchymal transition (EMT) contributed to the invasive phenotype and metastatic capacity. These results suggested that $CD26^+$ cells are cancer stem cells in colorectal cancer, and that CD26/DPPIV can be targeted for metastatic colorectal cancer therapy. More recently, significantly higher CD26 expression has been shown to be correlated with poorly differentiated tumor, late tumor node metastasis (TNM) stage (TNM stage III and stage IV), and development of metastasis (Lam et al., 2014). Moreover, a high CD26 expression level is a predictor of poor outcome after resection of CRC. These findings strongly suggest that CD26 may be a useful prognostic marker in patients with CRC. Other investigators demonstrated in a murine model that lung metastasis of CRC was suppressed by treatment with a DPPIV inhibitor (Jang et al., 2015). They showed a reduction of EMT markers, suggesting that the EMT status of the murine colon cancer cell line MC38 was at least in part affected by DPPIV inhibition, with a diminution in the growth of metastases. They also showed that DPPIV inhibition decreased the growth of lung metastases of colon cancer by downregulating autophagy, increasing apoptosis and arresting the cell cycle.

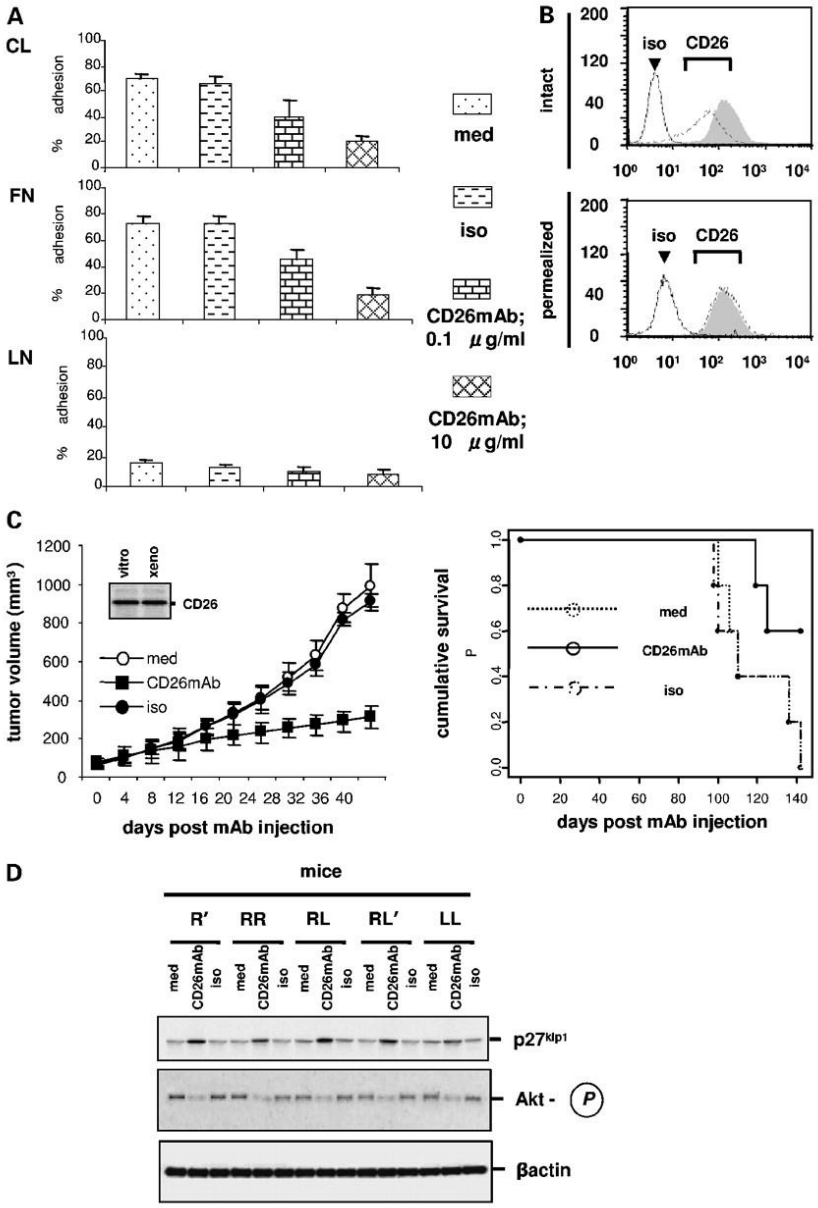

Figure 1. Antitumor effect of anti-CD26 mAb in mouse xenograft model of Caki-2, human renal carcinoma. (A) effect of anti-CD26 murine mAb (14D10) on cell adhesion to ECM. Caki-2 cells treated with medium only, anti-CD26 mAb, or isotype-matched control mAb were plated onto 60-mm dishes (3 x 10^6 per dish) coated with collagen I (*CL*), fibronectin (*FN*), or laminin (*LN*) and cultured for 21 hours. The

adhesive ability of cancer cells was expressed as the mean number of cells that had attached to the bottom surface of the dish. Columns, mean number of cells per field of view; bars, SE. Values for invasion were determined by calculating the average number of adhesive cells per mm^2 over three fields per assay and expressed as an average of triplicate determinations. Adhesive cells (%): adhesive cells / adhesive cells + nonadhesive cells. (B) Caki-2 cells were treated with anti-CD26 mAb on ice, or isotype-matched control mAb, followed by washing in ice-cold PBS twice and subsequently incubated at 37°C for 12 hours. Cells were collected and stained with FITC-conjugated anti-mouse IgG. Expression status of cell surface CD26 was analyzed by flow cytometry. To detect total CD26 level, including the internalized CD26 fraction, cell membrane permeabilization method was used. Filled histogram, positive control, which was incubated 30 minutes with anti-CD26 mAb. Open histogram, status of CD26 after treatment. (C) Caki-2 cells (1×10^6) were inoculated s.c. into the left flank of mice. CD26 expression of Caki-2 cells after tumor implantation into the mouse was similar to its level before tumor implantation. Mice were treated with intratumoral injection of PBS only (medium; $n = 5$), anti-CD26 mAb ($n = 5$), or isotype-matched control mAb ($n = 5$) on the day when the tumor mass became visible (5 mm in size). Tumor size and cumulative survival were monitored. (D) resected specimens were immediately frozen for whole-cell lysate preparation and lysed by lysis buffer. Protein (50 μg) was applied for SDS-PAGE and immunoblotting for $p27^{kip1}$, phosphorylated Akt, and β-actin. R,' RR, RL, RL,' and LL, names of mice in each treatment group. This figure is reprinted with permission from Inamoto T et al., *Clin Cancer Res* 12: 3470-7, 2006.

2.2.4. T-Anaplastic Large Cell Lymphoma and Acute T Cell Leukemia

We showed that treatment with anti-CD26 murine mAb inhibited adhesion of the human $CD30^+$ T-anaplastic large cell lymphoma cell line Karpas 299 to fibronectin. Furthermore, depletion of CD26 in Karpas 299 cells by siRNA decreased tumorigenesis and increased survival of SCID mice inoculated with these cells (Sato et al., 2005). In addition, we demonstrated that anti-CD26 murine mAb treatment of the CD26-positive Karpas 299 resulted in *in vitro* and *in vivo* anti-tumor activity. Administration of anti-CD26 mAb induced cell cycle arrest at the G1/S checkpoint, associated with enhanced p21 expression, and significantly enhanced survival of SCID mice inoculated with Karpas 299 cells by inhibiting tumor formation (Ho et al., 2001). Likewise, we showed that treatment with anti-CD26 murine mAb inhibited the growth of acute T cell leukemia cell line Jurkat transfected with CD26 through G1/S cell cycle arrest, associated with concurrent activation of the ERK signaling pathway and increased p21 expression (Ohnuma et al., 2002). These findings further

support a therapeutic approach involving targeted therapy against CD26 for selected hematological malignancies.

2.2.5. Gastrointestinal Stromal Tumor

Yamaguchi et al., comprehensively investigated gene expression profiles in surgical samples of untreated GIST of the stomach and small intestine. They found that the disease-free survival of patients with CD26-positive GIST of the stomach was worse than that of patients with CD26-negative GIST (Yamaguchi et al., 2008). Moreover, the postoperative recurrence rate of CD26-negative gastric GIST cases was as low as 2.0%. Meanwhile, CD26 expression was not associated with clinical outcome of small intestinal GIST. They concluded that CD26 is a significant prognostic factor of gastric GIST and may also serve as a therapeutic target (Yamaguchi et al., 2008).

2.2.6. Thyroid Carcinoma

The *BRAF*V600E mutation, which results in greater mitogen-activated protein kinase signaling output, is the most predominant oncogenic driver of thyroid cancer (Fagin and Wells, 2016). CD26 expression is upregulated in malignant thyroid tumors, and CD26 can be used as a malignancy marker in fine-needle aspiration cytology of thyroid nodules (de Micco et al., 2008). Recently, CD26, secretogranin V (SCG5) and carbonic anhydrase XII (CA12) are a three-gene signature that can distinguish malignant thyroid cancers, and useful for preoperative diagnosis of thyroid cancer (Zheng et al., 2015). More recently, the function of CD26 in thyroid cancer has been investigated. High CD26 was associated with extrathyroidal extension, *BRAF* mutation, and advanced tumor stage in papillary thyroid cancer (Lee et al., 2017). CD26 silencing by siRNA or treatment with DPPIV inhibitors significantly suppressed colony formation, cell migration, and invasion of thyroid cancer. CD26 expression was suggested to be involved in the transforming growth factor (TGF)-β signaling pathway. Furthermore, *in vivo* experiments revealed that treatment with the DPPIV inhibitor sitagliptin reduced tumor growth and xenograft TGF-β receptor I expression (Lee et al., 2017). These

investigators concluded that increased CD26 expression is associated with cellular invasion and more aggressive disease in papillary thyroid cancer, and targeting CD26/DPPIV may be a therapeutic strategy for CD26-expressing thyroid cancer.

2.2.7. Urothelial Carcinoma

Although there is an increasing number of biomarkers that have prognostic relevance to urothelial carcinoma (UC), factors involved in tumor progression remained largely unclear. Recently, by mining the datasets obtained from the Gene Expression Omnibus (GEO, NCBI, Bethesda) and focusing on the proteolysis pathway, Liang et al., discovered that mRNA level of CD26 is significantly upregulated in advanced-stage human UC and the upregulation of CD26 is most significantly associated with clinical aggressiveness of UC (Liang et al., 2017). CD26 is an independent prognostic biomarker for disease-specific survival and metastasis-free survival. Moreover, CD26 knockdown by shRNA resulted in a significantly decreased cell viability, proliferation, migration, and invasion in urothelial cell lines, J82 and RTCC-1 cells (Liang et al., 2017). These findings strongly suggest that CD26 plays a role in the aggressiveness of UCs, and can serve as a novel prognostic marker and therapeutic target.

2.2.8. Chronic Myeloid Leukemia

Chronic myeloid leukemia (CML) is a stem cell neoplasm characterized by the BCR/ABL1 oncogene. Herrmann et al., recently identified CD26/DPPIV as a novel, specific and pathogenetically relevant biomarker of $CD34^{+}CD38^{-}$ CML leukemic stem cell (LSC) (Herrmann et al., 2014). CD26 was not detected on normal stem cells or LSC in other hematopoietic malignancies. Correspondingly, $CD26^{+}$ LSC decreased to low or undetectable levels during successful treatment with imatinib. $CD26^{+}$ CML LSC engrafted NOD-SCID-IL-$2R\gamma^{-/-}$ (NSG) mice with $BCR/ABL1^{+}$ cells, whereas $CD26^{-}$ LSC from the same patients produced multilineage $BCR/ABL1^{-}$ engraftment. Moreover, targeting of CD26 by gliptins suppressed the expansion of $BCR/ABL1^{+}$ cells. These results

suggest that CD26 is a new biomarker and target of CML LSC, and inhibition of CD26/DPPIV may revert abnormal LSC function and support curative treatment approaches in this malignancy. More recently, Warfvinge et al., extensively defined the heterogeneity within the LSC population in chronic phase CML patients at diagnosis and following conventional tyrosine kinase inhibitor (TKI) treatment. Cell surface expression of the CML stem cell markers CD25, CD26, and IL1RAP was high in all subpopulations at diagnosis but downregulated and unevenly distributed across subpopulations in response to TKI treatment. The most TKI-insensitive cells of the LSC compartment could be captured within the $CD45RA^-$ fraction and further defined as positive for CD26 in combination with an aberrant lack of cKIT expression. These results expose a considerable heterogeneity of the CML stem cell population and propose a $Lin^-CD34^+CD38^{-/low}CD45RA^-cKIT^-CD26^+$ population as a potential therapeutic target for improved therapy response (Warfvinge et al., 2017).

2.3. Mechanisms of Action of YS110 for Cancer Treatment

We had developed a novel humanized anti-CD26 mAb, namely YS110. YS110 is a recombinant DNA-derived humanized mAb that selectively binds with high affinity to the extracellular domain of CD26. The antibody is an $IgG_1\kappa$ with a molecular weight of 144 kDa and was humanized via an *in silico* design based on the amino acid sequence of anti-human CD26 murine mAb (14D10), which inhibited tumor cell growth, migration and invasion, and enhanced survival of mouse xenograft models (Inamoto et al., 2006). YS110 is produced by fermentation in CHO (Chinese hamster ovary) mammalian cell suspension culture with the Glutamine Synthetase Expression System. *In vitro* pharmacologic evaluation of YS110 demonstrated its selective binding to human CD26 on a number of human cancer cell lines and tissues, with no apparent agonistic effect on human CD26-positive lymphocytes and no inhibition of DPPIV activity. Moreover, in the proof-of-concept (POC) studies using preclinical models, *in vivo* administration of YS110 resulted in inhibition of tumor cell growth,

migration and invasion, and enhanced survival of mouse xenograft models inoculated with RCC or MPM (Inamoto et al., 2006; Inamoto et al., 2007; Okamoto et al., 2014; Yamamoto et al., 2014). In addition to our robust *in vitro* and *in vivo* data on antibody-mediated dose-dependent tumor growth inhibition, YS110 exhibited excellent safety and pharmacological profiles in non-human primate models using single and repeated increasing intravenous doses. Other key safety findings were obtained from studies involving cynomolgus monkeys which express YS110-reactive CD26 molecules, with similar tissue distribution profiles and expression levels to human CD26. We therefore conducted the FIH phase 1 clinical trial of YS110 for patients with refractory MPM and other CD26-positive solid tumors (Angevin et al., 2017). Thirty-three heavily pre-treated patients with CD26-positive cancers including 22 MPM, 10 RCC and 1 UC underwent YS110 administration. Our FIH phase 1 study conclusively demonstrated that YS110 exhibits a favorable safety profile and substantial clinical activity in heavily pre-treated CD26-positive MPM and RCC patients who had previously progressed on conventional standard chemotherapies. Further clinical trial of YS110 for MPM is in progress worldwide (ClinicalTrials.gov, 2017).

The role of CD26 in cancer cell biology and the mechanisms of action of YS110 for cancer treatment have been reviewed in detail recently (Ohnuma et al., 2017). In brief, we summarize the mechanisms of action of YS110 as follows; (i) a direct cytotoxic effect on certain human CD26-positive cancer cell lines via antibody-dependent cell-mediated cytotoxicity (ADCC) (Inamoto et al., 2007), (ii) a direct anti-tumor effect through the induction of cell cycle arrest by induction of $p27^{kip1}$ and $p21^{cip1}$ expression (Inamoto et al., 2007; Hayashi et al., 2016), (iii) the nuclear translocation of CD26 molecules by internalization of the CD26-YS110 complexes to inhibit proliferation of tumor cells via suppression of *POLR2A* gene expression, a component of RNA polymerase II (Yamada et al., 2009; Yamada et al., 2013), and (iv) following internalization of the CD26-YS110 complexes, an inhibition of invasion and migration of tumor cells by decreased binding to the collagen/fibronectin microenvironment matrix (Inamoto et al., 2007). We are currently investigating other

mechanisms of action for the observed anti-tumor activity of YS110, especially focusing on the effects of YS110 on tumor immunology.

The FIH phase 1 clinical study of YS110 revealed that an increase in YS110 infusion dose was associated with decreased serum sCD26 level and DPPIV enzyme activity, particularly in cohorts 4-6 (2.0 to 6.0 mg/kg), with an approximately 80% decrease in these levels (Angevin et al., 2017). Although DPPIV inhibitors are clinically used as oral hypoglycemic agents (Drucker and Nauck, 2006), hypoglycemia was not observed during YS110 administration. Of note is the fact that greater than 80% inhibition of serum DPPIV activity was obtained 24 hours after oral administration of clinically available DPPIV inhibitors (drug information published by manufacturers of sitagliptin, vildagliptin, saxagliptin and *etc*.), a level of inhibition comparable to that seen in patients treated with YS110. Our current data would therefore indicate that YS110 therapy is tolerable in the clinical setting. Recent work has demonstrated the functional role of DPPIV-mediated post-translational modification of chemokines in regulating tumor immunity through its interaction with its substrate CXCL10 (Barreira da Silva et al., 2015). Barreira da Silvia et al., used *in vivo* tumor-transplant models to show that DPPIV inhibition reduced tumor growth through the preservation of bioactive CXCL10 in the tumor microenvironment (TME). In the normal physiological state, CXCL10 is rapidly degraded by DPPIV, resulting in decreased recruitment and migration of $CXCR3^+$ T cells into the TME. CXCR3 has been shown to be a functional receptor for CXCL10 (Proost et al., 2001). In contrast, DPPIV inhibition enhanced tumor rejection by preserving the full-length biologically active form of CXCL10, leading to increased trafficking of $CXCR3^+$ T cells into the TME (Ohnuma et al., 2015b). This anti-tumor response is potentiated in combination with other anti-tumor immunotherapeutic approaches including CpG adjuvant therapy, adoptive T cell transfer therapy and checkpoint blockade therapy (anti-CTLA-4 and anti-PD-1) (Barreira da Silva et al., 2015). In view of these recent findings, data from our current trial showing that serum DPPIV activity was decreased following treatment with YS110 in a dose-dependent manner would suggest that anti-tumor activity via DPPIV inhibition may constitute

yet another mechanism of action for the anti-tumor activity of YS110 (Ohnuma et al., 2015b).

Although the cellular and molecular mechanisms involved in CD26-mediated T cell activation have been extensively evaluated by our group and others (Morimoto and Schlossman, 1998; Ohnuma et al., 2008a; De Meester et al., 1999), potential negative feedback mechanisms to regulate CD26-mediated activation still remain to be elucidated. Utilizing human peripheral blood lymphocytes, we recently found that CD26-mediated costimulation induced the development of a population of human type 1 regulatory T (T_{reg}) cells from $CD4^+$ T cells with high level of IL-10 production and lymphocyte-activation gene 3 (LAG3) expression (Hatano et al., 2015). Other investigators have also reported that the $CD26^+CD39^-$ T_{reg} subset among $CD4^+$ T_{reg} exhibits high level of IL-10 expression (Hua et al., 2015). These findings strongly suggest that a specific subset of $CD26^+$ T cells plays a role in immune checkpoint system, and that the CD26 molecule may be a novel target for a therapeutic approach involving immune checkpoint blockade. We are currently investigating whether CD26-mediated signals are associated with the induction of immune checkpoint molecules in the tumor-infiltrating lymphocytes, and definitive data will be presented in the near future.

3. IMMUNE DISORDERS

3.1. Acute Graft-versus-Host Disease

GVHD is a severe complication and major cause of morbidity and mortality following allogeneic hematopoietic stem cell transplantation (alloHSCT) (Giralt, 2012). GVHD results from an immunobiological attack on target recipient organs by donor allogeneic T cells that are transferred along with the allograft (Blazar et al., 2012; Shlomchik, 2007). The pivotal role of donor-derived T cells in acute GVHD (aGVHD) is supported by the complete abrogation of GVHD following T cell depletion from the graft (Giralt, 2012; Tsirigotis et al., 2012). This approach remains

the most effective in preventing aGVHD. However, complications such as graft rejection, loss of GVL effect or increased opportunistic infections can occur. Although new approaches are being developed in the clinical setting for the prevention or treatment of GVHD (Blazar et al., 2012), in-depth understanding of the precise cellular mechanisms of human GVHD is necessary for more effective and less immunocompromising strategies to improve the clinical outcome of alloHSCT.

Costimulatory pathways are required to induce T cell proliferation, cytokine secretion and effector function following antigen-mediated T cell receptor activation (Rudd, 2010), and the important role of costimulatory pathways in transplant biology has been established (Markey et al., 2014). The most extensively studied pathways involve interactions between CD28 and the B7 molecules CD80 and CD86 (Rudd et al., 2009). Initial studies focused on the *in vivo* blockade of interactions between CD28 or cytotoxic T lymphocyte antigen 4 (CTLA4) and their B7 ligands, CD80 and CD86, using a CTLA4-immunoglobulin fusion protein (CTLA4-Ig) or B7-specific antibodies (Lenschow et al., 1992; Saito, 1998). Blockade of CD28 pathways may lead to profound immunosuppression (Blazar et al., 1997). CD26 is also associated with T cell signal transduction processes as a costimulatory molecule, as well as being a marker of T cell activation (Morimoto and Schlossman, 1998; Ohnuma et al., 2008a). In fact, patients with autoimmune diseases such as multiple sclerosis, Grave's disease, and RA have been found to have increased numbers of $CD4^+CD26^+$ T cells in inflamed tissues as well as in their peripheral blood (Eguchi et al., 1989; Gerli et al., 1996; Hafler et al., 1985; Mizokami et al., 1996), with enhancement of CD26 expression in these autoimmune diseases correlating with disease severity (Eguchi et al., 1989; Gerli et al., 1996; Muscat et al., 1994). Moreover, $CD26^{high}CD8^+$ T cells in humans belong to early effector memory T cells, and $CD26^{high}CD8^+$ T cells exhibited increased expression of granzyme B, TNF-α, IFN-γ and Fas ligand, and exerted cytotoxic effect with CD26-mediated costimulation (Hatano et al., 2013b). These findings implied that $CD26^+$ T cells play an important role in the inflammation process and subsequent tissue damage in such diseases, and suggested that

$CD26^+$ T cells belong to the effector T cell population. However, little is known about the effectiveness of CD26-targeting therapy on aGVHD.

To determine whether human $CD26^+$ T cells play a role in an animal model of inflammatory diseases mediated by human effector lymphocytes, we utilized a xenogeneic model of GVHD resulting from the adoptive transfer of human peripheral blood mononuclear cells (PBMCs) into NOD/Shi-*scid*, IL-2Rγ^{null} (NOG) mice (hu-PBL-NOG mice) (Ito et al., 2002). We first conducted a pathological analysis of x-GVHD target organs such as the skin, colon or liver in hu-PBL-NOG mice. The liver, colon and skin of hu-PBL-NOG mice were infiltrated with human $CD3^+$ mononuclear cells (MNCs), with associated organ destruction. Moreover, human $CD3^+$ MNCs reactive to anti-human CD26 Ab were readily visible in all evaluated samples in the liver, colon or skin of x-GVHD mice. The infiltrated $CD26^+$ cells were confirmed to be human $CD3^+$ T cells by co-staining analysis with flow cytometry. These results suggest that donor-derived human $CD26^+$ cells play a role in the pathogenesis of x-GVHD in our hu-PBL-NOG murine model.

We attempted to determine whether treatment with humanized anti-CD26 mAb could ameliorate disease progression and severity in our x-GVHD murine model. As comparison, the clinically available T cell costimulation blocking agent CTLA4-Ig (abatacept) was utilized (Genovese et al., 2005; Gribben et al., 1996). As shown in Figure 2A, anti-CD26 mAb (orange or red lines) or CTLA4-Ig (green or dark green lines) treatment of hu-PBL-NOG mice significantly increased overall survival, as compared with control IgG treatment (blue line). This life-prolonging effect of anti-CD26 mAb was observed at the same low dose as CTLA4-Ig (orange or green lines in Figure 2A, respectively). Moreover, GVHD-associated weight loss for up to 4 weeks post cell inoculation was not significantly different between anti-CD26 mAb and CTLA4-Ig treatment groups (orange or green lines in Figure 2B). These data strongly suggest that anti-CD26 mAb treatment is a promising novel therapeutic agent for x-GVHD, with efficacy comparable to CTLA4-Ig.

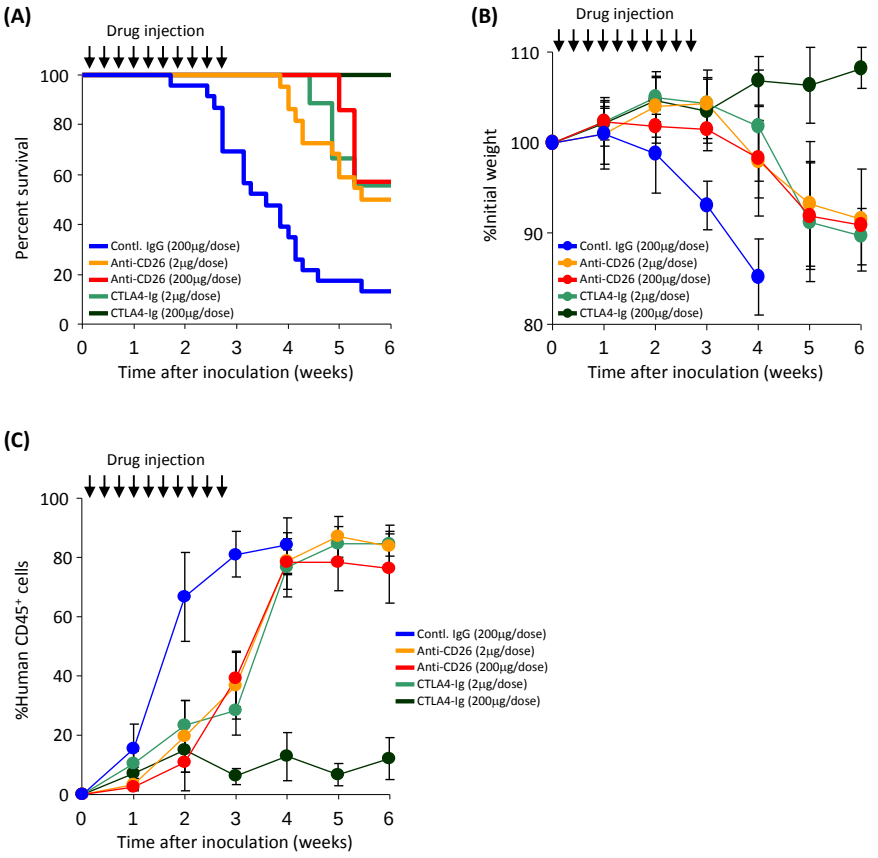

Figure 2. Anti-CD26 mAb treatment reduces x-GVHD-related lethality, and weight loss in hu-PBL-NOG mice without rejection of donor-derived human lymphocytes. After 1 day of inoculation of human PBMCs, Hu-PBL-NOG mice were injected intraperitoneally with humanized anti-CD26 mAb, CTLA4-Ig, or isotype IgG control at indicated doses in 200 μl of sterile phosphate-buffered saline (PBS), and then were injected thrice weekly for a total of 10 doses to assess potency in preventing x-GVHD. (A) Kaplan–Meier survival curves for mice receiving PBMC plus control IgG (200 μg/dose, blue line, n = 23), low dose anti-CD26 mAb (2 μg/dose, orange line, n = 24) (*P* = 0.0001 vs. control IgG group), high dose anti-CD26 mAb (200 μg/dose, red line, n = 7) (*P* = 0.0006 vs. control IgG group), low dose CTLA4-Ig (2 μg/dose, green line, n = 9) (*P* = 0.0005 vs. control IgG group), or high dose CTLA4-Ig (200 μg/dose, dark green line, n = 6) (*P* = 0.0008 vs. control IgG group). (B) Average weight (percentage ± standard deviation (SD) of initial) for mice surviving on a given day for different groups of mice as shown in (A). (C) Time course changes of average percentage (± SD) of human $CD45^+$ lymphocytes in peripheral blood in mice receiving PBMC

plus control IgG (200 μg/dose, blue line, n = 23), low dose anti-CD26 mAb (2 μg/dose, orange line, n = 24), high dose anti-CD26 mAb (200 μg/dose, red line, n = 7), low dose CTLA4-Ig (2 μg/dose, green line, n = 9), or high dose CTLA4-Ig (200 μg/dose, dark green line, n = 6). This figure is reprinted with permission from Hatano R et al., *Br J Haematol* 162: 263-77, 2013.

We next analyzed circulating human lymphocytes in the peripheral blood of hu-PBL-NOG mice receiving anti-CD26 mAb or CTLA4-Ig. As shown in Figure 2C, at 3 weeks after inoculation, human lymphocyte level was increased in peripheral blood of mice receiving anti-CD26 mAb at both low dose and high dose (orange and red lines of Figure 2C), comparable to low dose CTLA4-Ig (green line of Figure 2C). On the other hand, in mice receiving high dose CTLA4-Ig, most of the inoculated human PBMCs were rejected (dark green line in Figure 2C). These data indicate that while treatment with increasing doses of CTLA4-Ig resulted in the absence of x-GVHD development, graft rejection did occur, potentially analogous to findings in a clinical trial with a new CTLA4-Ig agent, belatacept (Vincenti et al., 2010). On the other hand, increasing doses of anti-CD26 mAb resulted in no graft rejection in hu-PBL-NOG mice with prolonging survival as compared to hu-PBL-NOG mice receiving control IgG.

We next examined human CD26 expression level in the peripheral blood of hu-PBL-NOG mice receiving anti-CD26 mAb or CTLA4-Ig. Higher expression level of human CD26 on both $CD4^+$ and $CD8^+$ human lymphocytes in the peripheral blood of hu-PBL-NOG mice receiving control IgG or CTLA4-Ig was observed from 1 to 3 weeks after inoculation. On the other hand, in hu-PBL-NOG mice receiving anti-CD26 mAb, neither $CD4^+$ nor $CD8^+$ T cells expressed CD26 from 1 to 3 weeks. At 3 weeks after inoculation, very weak expression of human CD26 was observed on $CD4^+$ or $CD8^+$ cells in the peripheral blood of hu-PBL-NOG mice receiving anti-CD26 mAb. Taken together, these data suggest that decreased number of $CD26^{high}$ effector T cells may be responsible for the relative absence of x-GVHD development in mice receiving anti-CD26 mAb.

Since $CD26^{high}$ effector lymphocytes have high capacity for migration into inflamed tissues, we examined liver tissues of hu-PBL-NOG mice for

donor lymphocyte infiltration. In x-GVHD mice receiving control IgG, liver damage was observed with infiltration of human $CD4^+$ or $CD8^+$ MNCs. Moreover, slight inflammation of the portal duct areas in the liver was observed in mice receiving CTLA4-Ig. On the other hand, infiltration of human T cells in the liver was barely detected in mice receiving anti-CD26 mAb. In addition to the pathological changes seen in the liver, significant elevation of serum alanine aminotransferase (ALT) activity was observed in mice receiving control IgG or CTLA4-Ig, while that of mice receiving anti-CD26 mAb was found to be near normal level. Along with lymphocyte infiltration in the GVHD target tissues, the mRNA level of effector cytokines of donor-derived human $CD4^+$ or $CD8^+$ cells in the liver of hu-PBL-NOG mice receiving anti-CD26 mAb was decreased compared to those of hu-PBL-NOG mice receiving control IgG. Taken together, these results indicate that anti-CD26 treatment in hu-PBL-NOG mice ameliorated liver GVHD by decreasing production of proinflammatory cytokines of donor-derived human lymphocytes as well as inhibiting lymphocyte infiltration in the liver.

Since aGVHD and GVL effects are immune reactions highly linked to each other (Wu and Ritz, 2009; Zorn et al., 2002), we evaluated the potential influence of anti-CD26 mAb treatment on GVL effect. NOG mice transplanted with P815 cells along with human PBMCs and control IgG showed minimal signs of tumor growth in the inoculated region, but all mice died around 4 weeks after inoculation due to x-GVHD. On the other hand, mice inoculated with P815 along with human PBMC and anti-CD26 mAb exhibited enhanced survival rate with minimal evidence of GVHD. Importantly, mice in this group showed significantly slow initial tumor growth, suggesting the preservation of GVL effect more than CTLA4-Ig treatment. We examined the expression level of effector cytokines of human $CD8^+$ T cells isolated from the spleens of hu-PBL-NOG mice at 2 weeks after transplantation. Our data suggest that the GVL effect of cytotoxic effector function occurring at the early time period prior to manifestation of x-GVHD was preserved in hu-PBL-NOG mice receiving anti-CD26 mAb, compared to those in hu-PBL-NOG mice receiving CTLA4-Ig. In conclusions, CD26-mediated T cell activation

appears to play a significant role in GVHD. Since full suppression of x-GVHD with interventional therapies is currently a difficult challenge, our data demonstrating that control of x-GVHD can be achieved by modulating $CD26^{high}$ T cells with anti-CD26 mAb are potentially important clinically. Our work also suggests that anti-CD26 mAb treatment may be a novel therapeutic approach for GVHD in the future.

3.2. Chronic Graft-versus-Host Disease

On the basis of differences in clinical manifestations and histopathology, GVHD can be divided into acute and chronic forms (Blazar et al., 2012). aGVHD and chronic GVHD (cGVHD) are traditionally diagnosed primarily by time of onset, with cGVHD occurring after day 100 of transplantation (Deeg et al., 1997). However, cGVHD has distinct clinicopathologic features and is often diagnosed based on these features regardless of time of onset, being characterized by cutaneous fibrosis, involvement of exocrine glands, hepatic disease, and obliterative bronchiolitis (OB) (Filipovich, 2008; Socie and Ritz, 2014). OB, characterized by airway blockade, peribronchiolar and perivascular lympho-fibroproliferation and obliteration of bronchioles, is a late-stage complication of cGVHD (Chien et al., 2010). Patients diagnosed with OB have a 5-year survival rate of only 10 to 40%, compared to more than 80% of patients without OB (Dudek et al., 2003; Nakaseko et al., 2011). Furthermore, while multiple strategies to control cGVHD involving T cell depletion from the graft or global immunosuppression have been developed, cGVHD is still a common clinical outcome in many alloHSCT patients (Socie and Ritz, 2014; Zeiser and Blazar, 2016). In addition, immunosuppression potentially abrogates the GVL effect, associated with increased relapses following alloHSCT (Champlin et al., 1999). Novel therapeutic approaches are thus needed to control cGVHD without eliminating the GVL effect.

Since our aGVHD model described in the preceding section succumbed to aGVHD around 4 weeks after transplantation of human adult

PBL, this early-onset model of aGVHD does not permit the assessment of longer term consequences of interventional therapies, such as their effect on the development of OB, a form of cGVHD of the lung. In contrast to adult PBL, human cord blood (HuCB) lymphocytes have been reported to be immature, predominantly consisting of $CD45RA^+$ naive cells (Kobayashi et al., 2004). We previously showed that, while all HuCB CD4 T cells constitutively express CD26, CD26-mediated costimulation was considerably attenuated in HuCB CD4 T cells, compared to the robust activation via CD26 costimulation of adult PBL (Kobayashi et al., 2004). Based on these findings, we hypothesized that HuCB naive CD4 T cells gradually acquire a xenogeneic response via attenuated stimulatory signaling with indolent inflammation in the target organs, leading eventually to chronic inflammatory changes. We therefore succeeded in developing a humanized murine pulmonary cGVHD model utilizing NOG mice as recipients and HuCB as donor cells (Ohnuma et al., 2015a), and overcame the limitations seen in the humanized murine aGVHD model such as vigorous activation of all engrafted T cells and extensive loss of B cell maturation and activation (Shultz et al., 2012). Whole CB transplant mice exhibited clinical signs/symptoms of GVHD as early as 4 weeks post transplant, and demonstrated significantly decreased survival rate. The lung of whole CB transplant mice showed perivascular and subepithelial inflammation and fibrotic narrowing of the bronchiole. Skin of whole CB transplant mice manifested fat loss, follicular drop-out and sclerosis of the reticular dermis in the presence of apoptosis of the basilar keratinocytes while the liver exhibited portal fibrosis and cholestasis. These findings indicate that whole CB transplant mice develop pulmonary cGVDH as well as concomitant active GVHD in skin and liver. Taken together, our data demonstrate that the lung of whole CB transplant mice exhibits OB as manifestation of pulmonary GVHD (Ohnuma et al., 2015a).

Utilizing this model, we identified IL-26 as a key effector cytokine inducing transplant-related obliterative bronchiolitis. Lung of HuCB mice exhibited obliterative bronchiolitis with increased collagen deposition and predominant infiltration with human $IL\text{-}26^+CD26^+CD4$ T cells. Moreover, although IL-26 is absent from rodents, we showed that IL-26 increased

collagen synthesis in fibroblasts and promoted lung fibrosis in a murine GVHD model using *IL-26* transgenic mice. *In vitro* analysis demonstrated a significant increase in IL-26 production by HuCB CD4 T cells following CD26 costimulation, whereas Ig Fc domain fused with the N-terminal of caveolin-1 (Cav-Ig), the ligand for CD26, effectively inhibited production of IL-26. Administration of Cav-Ig before or after onset of GVHD impeded the development of clinical and histologic features of GVHD without interrupting engraftment of donor-derived human cells, with preservation of the GVL effect (Ohnuma et al., 2015a). These results therefore provide proof of principle that cGVHD of the lungs is caused in part by IL-26^{+}CD26^{+}CD4 T cells, and that blockade of CD26-caveolin-1 interaction by Cav-Ig or YS110 could be beneficial for cGVHD prevention and therapy.

3.3. Rheumatoid Arthritis

RA is a chronic, inflammatory autoimmune disease that primarily affects the joints, but also has systemic symptoms. RA is characterized by progressive invasion of synovial fibroblasts into the articular cartilage and erosion of the underlying bone, followed by joint destruction (Asif Amin et al., 2017). Several reports on RA patients have shown that concentration of soluble CD26 and DPPIV enzyme activity were significantly decreased in both synovial fluid and serum compared with osteoarthritis patients or healthy donors (Busso et al., 2005; Buljevic et al., 2013; Cordero et al., 2015). Cordera et al., studied serum levels of IL-12, IL-15, and soluble CD26 from 35 patients with active and inactive RA as well as those of healthy controls (Cordero et al., 2001). Patients' sera had higher IL-12 and IL-15 levels, and the level of soluble CD26 was inversely correlated with the number of swollen joints. These findings suggest that these cytokines and CD26 are associated with the inflammation and immune activity in RA. A number of proinflammatory peptides are supposed to be involved in the pathogenesis of RA, and have their biological activity controlled by limited proteolysis mediated by DPPIV and DPPIV-like hydrolytic activity (Wolf

et al., 2008). Several investigators have demonstrated that SDF-1α (CXCL12), a substrate of DPPIV, is a crucial mediator controlling the influx of lymphocytes and monocytes/macrophages into the inflamed synovium of RA and thus triggering joint destruction (Kim et al., 2007; Sromova et al., 2010). DPPIV regulates neuropeptide Y (NPY) and vasoactive intestinal peptide (VIP), recently implicated in RA (Buljevic et al., 2013). However, involvement of DPPIV enzyme activity in the pathology of RA remains controversial. Using CD26/DPPIV-KO mice, Busso et al., demonstrated that damage severity, as indicated by synovial thickness, knee-joint inflammation, and histological grading, was more severe in CD26/DPPIV-KO mice than in wild-type control mice in antigen- and collagen-induced arthritis models (Busso et al., 2005). The authors concluded that decreased circulating CD26/DPPIV levels in arthritis increased the intact active form of SDF-1, associated with increased numbers of CXCR4 (SDF-1 receptor)-positive cells infiltrating arthritic joints. On the other hand, using a type II collagen-induced or alkyldiamine-induced rat model of arthritis, Tanaka et al., showed that treatment with any DPPIV inhibitors examined reduced hind paw swelling, an indicator of disease severity (Tanaka et al., 1997; Tanaka et al., 1998).

In contrast with the decrease in the levels of soluble CD26 in the synovial fluid and serum, patients with active RA displayed higher percentage of peripheral blood $CD26^{+}CD4^{+}$ T cells than inactive RA and control subjects (Muscat et al., 1994; Cordero et al., 2015). RA synovial fluid contained lower percentages of whole $CD26^{+}$ T cells compared with peripheral blood, while the percentage of $CD26^{high}$ T cells in synovial fluid of RA was markedly increased compared with peripheral blood of RA patients and healthy subjects (Mizokami et al., 1996). These findings suggest that $CD26^{+}$ T cells induce the inflammation and tissue destruction characteristic of RA by migrating to and being active in the rheumatoid synovium. We described $CD26^{+}$ T cells infiltrating the rheumatoid synovium using immunohistochemical studies (Ohnuma et al., 2006). We also noted high expression of caveolin-1 in the rheumatoid synovium and synoviocytes. These data suggest that the CD26-caveolin-1-mediated upregulation of CD86 on activated monocytes in addition with CD26-

mediated signal transduction in T cells leads to antigen specific T cell activation in RA, and blockade of this CD26-caveolin-1 interaction by YS110 may be useful for suppressing the immune system in RA.

3.4. Inflammatory Bowel Diseases

In patients with inflammatory bowel diseases (IBD) such as Crohn's disease or ulcerative colitis, $CD26^+$ T-cells and DPPIV activity in serum were examined (Hildebrandt et al., 2001; Rose et al., 2002). In these studies, while the DPPIV activity was reduced in patients with IBD, the number of $CD25^+CD26^+$ T-cells in the peripheral blood was increased in patients with IBD. Moreover, other investigators recently reported that $CD26^{high}$ T cells contain T_H17 cells, and that $CD26^{high}$ T_H17 cells are enriched in the inflamed tissue of IBD patients (Bengsch et al., 2012). In addition, Kappeler et al., showed that activated perforin mRNA expressing T cells are present in close proximity to the intestinal epithelial cells in active stages of ulcerative colitis and Crohn's disease (Kappeler et al., 2000), suggesting an important role of cytotoxic cells in the pathogenesis of IBD. We recently showed that $CD26^{high}CD8^+$ T cells exhibit increased expression of granzyme B, TNF-α, IFN-γ and Fas ligand, and exert cytotoxic effect with CD26-mediated costimulation (Hatano et al., 2013b). Taken together, these data indicate that CD26 may be potentially important for the pathophysiology of IBD, and appears to be a useful therapeutic target for IBD.

3.5. Middle East Respiratory Syndrome Coronavirus

MERS-CoV was first identified in a 60-year-old patient in June 2012 who presented with acute pneumonia, followed by acute respiratory distress syndrome and renal failure with a fatal outcome (Zaki et al., 2012). Between 2012 and September 2017, 2080 laboratory-confirmed cases of MERS-CoV infection were reported to the World Health Organization

(WHO), which has notified of at least 722 deaths (around 35% fatality rate) related to MERS-CoV since September 2012 (WHO, 2017). Efforts to develop effective preventive and therapeutic intervention strategies are currently ongoing. Several treatment modalities have been investigated to develop effective therapies against MERS-CoV, including interferon, ribavirin, cyclosporin A, protease inhibitors, convalescent plasma and immunoglobulins. Several promising anti-MERS-CoV therapeutic agents have recently been reviewed extensively (Mo and Fisher, 2016), while broad spectrum antiviral agents might not be sufficient to treat severe MERS-CoV patients because of its limited effective therapeutic window of opportunity (Zumla et al., 2016).

An alternative approach using prophylactic regimens would be theoretically suitable to limit the spread of MERS-CoV. This scenario includes MERS vaccine and neutralizing MERS-CoV-specific mAb (Zumla et al., 2016). The MERS-CoV genome encodes for 16 non-structural proteins (nsp1-16) and 4 structural proteins, the spike (S), envelope (E), membrane (M), and nucleocapsid (N) (van Boheemen et al., 2012). The viral structural proteins, S and N, show the highest immunogenicity (Agnihothram et al., 2014). While both S and N proteins induce T-cell responses, neutralizing antibodies are almost solely directed against the S protein, with the receptor binding domain (RBD) being the major immunodominant region (Mou et al., 2013). These great challenges have been extensively reviewed previously (Zumla et al., 2016).

Recent reports indicated that the spike protein S1 of MERS-CoV is required for viral entry into human host cells (Gierer et al., 2013; Lu et al., 2013), using CD26/DPPIV as a functional receptor (Raj et al., 2013). Inhibiting virus entry into host cells could also be achieved by targeting the host receptor CD26/DPPIV. While inhibitors of the CD26/DPPIV enzymatic activity are used clinically to treat type 2 diabetes patients, commercially available DPPIV inhibitors would not serve this purpose since these agents have been shown not to inhibit binding of the RBD of MERS-CoV to CD26/DPPIV (Raj et al., 2013). We previously showed that human CD26 is a binding protein for ADA (Kameoka et al., 1993). Currently, it is known that there are two isoforms of ADA, ADA1 and

ADA2. ADA1 is particularly present in lymphocytes and macrophages, while ADA2 is found predominantly in the serum and other body fluids including pleural effusion (Gakis, 1996). CD26/DPPIV binds to ADA1, but not ADA2 (Kameoka et al., 1993). The crystal structure of CD26/DPPIV and the S protein of MERS-CoV allowed for visualization of the interacting amino acid in both proteins. However, our *in vitro* experiments showed that blockade of MERS-CoV binding to CD26/DPPIV by ADA1 is incomplete (Ohnuma et al., 2013). Therefore, mAb-mediated blocking CD26/DPPIV binding to the RBD of MERS-CoV needs to be developed.

CD26/DPPIV, a host receptor for MERS-CoV, is conserved among different species such as bats and humans, partially explaining the large host range of MERS-CoV (Mohd et al., 2016). In addition to being widely expressed in most cell types including T lymphocytes, bronchial mucosa or the brush border of proximal tubules, CD26/DPPIV exists in systemic circulation as soluble form (Ohnuma et al., 2008b). This distribution of CD26 may play a role in the systemic dissemination of MERS-CoV infection in human (Drosten et al., 2013). Therefore, an effective therapy for MERS-CoV is needed not only to block the entry of MERS-CoV into such CD26-expressing organs as the respiratory system, kidney, liver or intestine, but also to eliminate circulating MERS-CoV. In this regard, manipulation of CD26/DPPIV levels or the development of inhibitors that target the interaction between the MERS-CoV S1 domain and its receptor may provide therapeutic opportunities to combat MERS-CoV infection. More recently, the RBD in the S protein was mapped to a 231-amino acid fragment of MERS-CoV S proteins (residues 358-588) (Mou et al., 2013).

We have recently mapped MERS-CoV S protein-binding regions in human CD26 molecule and demonstrated that anti-CD26 mAbs, which had been developed in our laboratory, effectively blocked the interaction between the spike protein and CD26, thereby neutralizing MERS-CoV infectivity. To determine the specific CD26 domain involved in MERS-CoV infection, we chose six different clones of anti-CD26 mAbs (4G8, 1F7, 2F9, 16D4B, 9C11, 14D10), and the humanized anti-CD26 mAb YS110, which recognize 6 distinct epitopes of the CD26 molecule (Dong

et al., 1998; Inamoto et al., 2007), to conduct MERS-CoV S1-Fc (S1 domain of MERS-CoV fused to the Fc region of human IgG) binding inhibition assays (Ohnuma et al., 2013). 2F9 inhibited fully binding of MERS-CoV S1-Fc to JKT-hCD26WT (Jurkat cells transfected with full-length human CD26/DPPIV), while other anti-CD26 mAbs demonstrated certain levels of inhibition (1F7, or YS110) or no significant inhibition (4G8, 16D4B, 9C11 or 14D10). These results strongly suggest that the anti-CD26 mAb 2F9 has better therapeutic potential than recombinant MERS-CoV S1-Fc in preventing viral entry into susceptible cells, and that 1F7 or YS110 also blocks MERS-CoV infection.

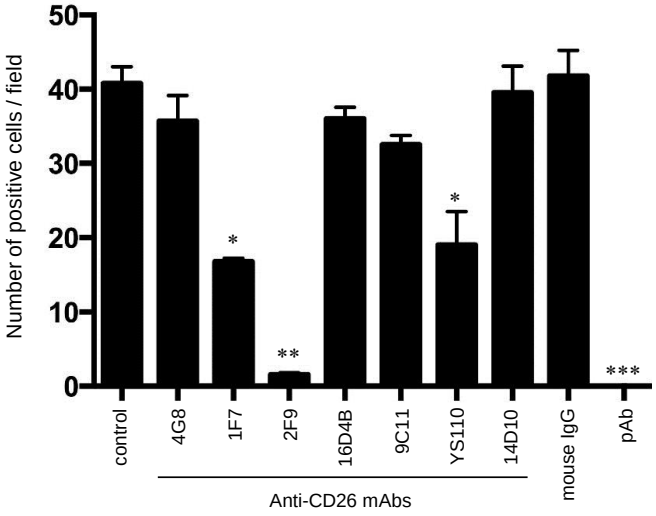

Figure 3. Inhibition of MERS-CoV infection by the anti-CD26 mAb. Huh-7 cells were preincubated with normal mouse IgG, various anti-CD26 mAbs (4G8, 1F7, 2F9, 16D4B, 9C11, or 14D10), humanized anti-CD26 mAb (YS110), or anti-CD26 goat polyclonal antibody (pAb) at a concentration of 40 μg/ml for 0.5 h prior to MERS-CoV virus inoculation (1 h), all at room temperature. Mock-incubated cells (control) were used as controls. Following incubation at 37°C for 8 h, infected cells were detected by immunofluorescence using anti-SARS-CoV NSP4 antibodies that are cross-reactive for MERS-CoV, and infection was quantified as the number of anti-SARS-CoV NSP4-positive cells. Two independent experiments were performed, and data from one representative experiment are shown. Error bars indicate SEMs (two-tailed Student's *t* test; *, **, or ***, $P < 0.05$ versus control. This figure is reprinted with permission from Ohnuma K et al., *J Virol* 87: 13892-9, 2013.

Moreover, we have characterized the CD26 epitope involved in MERS-CoV S1-Fc binding to CD26 through the use of various CD26 mutants with deletion in the C-terminal extracellular region, since CD26 is a type II transmembrane protein (Ohnuma et al., 2008a). Our biological experiments on binding regions of CD26 to MERS-CoV using mAbs showed results comparable to those obtained from crystal structure analysis (Lu et al., 2013; Wang et al., 2013). Our observations strongly suggest that the main binding regions of CD26 to MERS-CoV appear to be close to the 358th amino acid recognized by 2F9, and the regions of CD26 defined by 1F7 and YS110 (248-358th amino acids) are also partially involved in MERS-CoV binding.

To determine whether treatment with anti-CD26 mAbs 2F9 as well as 1F7 and YS110 could inhibit MERS-CoV infection, susceptible cells, Huh-7, were pre-incubated with various anti-CD26 mAbs prior to inoculation with the virus (Ohnuma et al., 2013). In this experimental system, infection was almost completely blocked by 2F9 but not by control IgG or several other anti-CD26 mAbs recognizing other epitopes (4G8, 16D4B, 14D10 or 9C11) (Figure 3). Moreover, the anti-CD26 mAbs 1F7 and YS110 considerably inhibited MERS-CoV infection of Huh-7 cells (Figure 3). These results demonstrate that 2F9 inhibits MERS-CoV entry, and therefore can potentially be developed as a preventive or therapeutic agent for MERS-CoV infection in the clinical setting. More importantly, the humanized anti-CD26 mAb YS110 has been evaluated in patients with CD26-expressing cancers in our FIH phase 1 clinical trial. Since no apparent adverse effects of YS110 have been reported besides transient and tolerable injection reactions, and moreover, the level of circulating soluble CD26 in the serum is decreased following YS110 administration (Angevin et al., 2017), YS110 may be an immediate therapeutic candidate for clinical use as potential treatment for MERS-CoV infection.

CLOSING REMARKS

CD26 is a multifunctional protein with known DPPIV enzyme activity. CD26 is expressed on various tumors including MPM. Although the exact role of CD26/DPPIV in various cancers remains to be elucidated, CD26 serves as a prognostic marker in multiple tumors such as CRC, GIST, thyroid carcinoma and UC. Moreover, in several human malignancies including MPM, CRC and CML, CD26/DPPIV expression is reported to be a marker of cancer stem cells. Our FIH phase 1 clinical trial of YS110 demonstrates that YS110 therapy exhibits a favorable safety profile and results in encouraging disease stabilization in a number of patients with advanced/refractory CD26-expressing MPM and RCC, and further clinical testing of YS110 for MPM is being conducted worldwide. Given the potential role of CD26 surface expression in cancer biology, YS110 therapy may also influence tumor growth through its potential effect on the cancer stem cells of selected tumors.

Besides being a marker of T cell activation, CD26 is also associated with T cell signal transduction processes as a costimulatory molecule and has an important role in T cell biology and overall immune function. Since CD26/DPPIV has a multitude of biological functions in human tumor cells and immune system, further detailed understanding of the role of this molecule in various clinical settings may lead potentially to novel therapeutic approaches not only for MPM but also for other refractory cancers, immune disorders and MERS-CoV infection.

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産業医学のプリンシプル～大切なこと

―産業医学振興財団40周年記念誌―

「産業医学ジャーナル」編集委員会 編

公益財団法人
産業医学振興財団
The Occupational Health Promotion Foundation

石綿関連疾患研究の現在

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石綿関連疾患とは、石綿肺、石綿肺癌、中皮腫、良性石綿胸水、びまん性胸膜肥厚を称する。2005年の兵庫県尼崎市のクボタ神崎工場周辺の環境曝露によって発症した中皮腫症例が多発したことから日本における研究が進展したが、古典的なじん肺の一種である石綿肺の研究は昭和15年から大阪泉南を中心として行われており、それ以降の研究が石綿肺癌の認定基準に貢献した。

1．石綿肺

石綿肺の定義は1986年のアメリカ呼吸器学会(ATS)[1]において、病理組織学的に細気管支周囲から末梢への線維化を認める肺線維症と定義されたが、Helsinki Criteria 1997[2]にて、“よくふくらませた肺において、びまん性の間質性線維化を示す組織切片上1cm^{2}の領域に2個以上の石綿小体か、被覆されていない石綿繊維をみること”と改正された。この改正によって、usual interstitial pneumonia(UIP)の組織像をとる肺線維症他も石綿小体数が基準以上認められたならば石綿肺と診断することになった。

しかし、2010年アメリカ呼吸器病理学会は石綿肺の定義を1986年のATSの基準であった細気管支周囲から末梢肺への線維化と細気管支周囲の石綿小体あるいは繊維の沈着すなわち病理組織学的にperibronchial fibrosisを証明する古典的な石綿肺のみを石綿肺と診断するとした[3]。このような症例では胸部HRCTにおいて線維化病変の軽い部位にperibronchial fibrosisを示すsubpleural curvilinear linesを認め、そうでない間質性肺炎との鑑別に有意な所見である[4]。典型的な石綿肺症例では肺組織内の石綿小体数は100万本/g以上の大量の石綿曝露を確認できるが、そうでない症例では多くても数万本/g程度と少ない[4]。日本でも最近増加しているUIP/IPFや喫煙によって生ずるCombined Pulmonary fibrosis and emphysema(CPFE)等との鑑別が問題であるが、この2010年の基準を参考に、胸部HRCTにおけるsubpleural curvilinear lines等の画像所見が石綿肺診断のために重要であるとする研究が継続されている[5]。

2．石綿肺癌

石綿によって発生する肺癌は石綿肺が原因である(asbestosis-cancer hypothesis)と考えられたこともあったが、現在では石綿自体(asbestos-cancer hypothesis)により発生すると考えられている。石綿による肺癌発生は石綿曝露と量 - 反応関係にあり、曝露量が多ければ多いほど発生頻度が増加する。石綿高濃度曝露によって発症する石綿肺の程度が強いほど肺癌発生頻度が高くなることは南アフリカのクロシドライト鉱山労働者の剖検データ[8]が証明している。Helsinki Criteia1997[2]によれば肺癌発生頻度が2倍になる石綿曝露量は25石綿繊維/ml×年である。具体的には肺乾燥重量1gあたり、石綿小体数が5,000～15,000本である。石綿単独曝露による肺癌発生は中皮腫ほど高くはないが、喫煙による肺癌発生頻度は相乗効果を示す。最近の調査のメタアナリシス[6]では、非喫煙者で非石綿曝露者を対照とした場合の肺癌発生のodds ratio(OR)は、非喫煙者で石綿曝露が1.70、喫煙者で非石綿曝露者が5.65、喫煙者の石綿曝露者が8.70であり、石綿単独曝露では肺癌発生危険率が低いと報告されている。Hendersonら[7]による多数の文献レビューによるとすべての肺癌の中の

4～12%が石綿関連肺癌であると報告している。

石綿による肺癌のバイオマーカーとして、2p16染色体領域のコピー数の減少とアレルの欠失(allelic imbalance；AI)[9]、9p21.3/p16の欠失、19p13の欠失等が報告[10]されている。また、9q33.1のAIは石綿曝露者でより頻度が高く、石綿曝露との量 - 反応関係があると報告[11]されている。最近では、7q32.1に位置するC7orf54遺伝子のsingle nucleotide polymorphism (SNP)が石綿曝露と最も強い関連があると報告され[12]、22q13.31染色体にあるMIRLET7Bのpolymorphismが肺癌発生の予測因子となるという報告[13]もある。石綿肺癌のバイオマーカーについてはそのほかの遺伝子の分子生物学的な更なる検討が必要である。

3．中皮腫

日本においても欧米においても中皮腫の診断には10%以上誤診が含まれることが報告されていたが、免疫染色法や中皮腫の遺伝子異常を基にした確定診断法が進歩している。

（1）診断

①病理組織あるいは細胞診断の免疫染色法により、カルレチニン（calretinin）が使用されるようになり、肺癌等との鑑別診断が比較的容易になった。その後、鑑別マーカーとしてD2-40等が使用されるようになった。新たな中皮腫の変異マーカーとして、BRCA-1-associated protein 1(BAP-1)は肺癌の場合には陰性化することはないが、中皮腫では陰性化した場合の診断意義が高いと報告されている[14]。

一方、肉腫型中皮腫の診断にはケラチン(keratin)の陽性が重要であり、CAM5.2あるいはAE1/AE3の陽性が真の肉腫との鑑別に有用である。しかし、ケラチン陰性の肉腫型中皮腫も約10%あることも報告されており、これらの鑑別について新たな研究が必要となっている。良性石綿胸水との鑑別にはFISH法を用いることにより、中皮腫に特異的な変異であるp16遺伝子のdeletionの頻度により肉腫型中皮腫と線維性胸膜炎の鑑別が可能となった[15]。

②胸水マーカーとして、胸水ヒアルロン酸が10万ng/mL以上であれば、中皮腫の可能性が高いという報告は1980年代から指摘されていた。2000年以降になり新たな胸水陽性マーカーとして、soluble mesothelin related peptides(SMRP)[16]あるいはSecretory leukocyte peptidase inhibitor(SLPI)[17]がヒアルロン酸と同等の診断マーカーとして報告されている。

（2）治療

化学療法としては2003年に胸膜中皮腫に対してCDDP+pemetrexedが有効であると報告されて以来、有効な治療法の報告がなかったが、近年新たな治療薬が開発され、その効果が期待されている。

免疫療法として、抗PD-1抗体であるpembrolizumab[18]およびnivolumab[19]が1次治療無効例にも有効でresponse rateは約30%であることが報告された。その他抗CD26人化抗体やREICを用いた遺伝子治療の臨床試験が開始され、その効果が期待されている。

手術療法としては1990年代から胸膜肺全摘術(EPP)が完全治癒を期待できる手術法として全世界で施行されていたが、術後30日以内の合併症による死亡率が高いため、壁側胸膜切除術に臓側胸膜剥離術を加える肺温存療法(P/D)が施行されるようになった。近年この両者の予後はほぼ同等であることが明らかとなり、合併症が少なく、肺が残存するため術後管理が容易なP/Dが胸膜中皮腫手術療法の主流になっている[20]。

4．良性石綿胸水

新たな診断基準の設定

本疾患の定義は1982年に提唱された後、他疾患との鑑別のための新たな診断基準がないのが現状である。平成19年度厚生労働科学研究の報告書[21]では、**表1**のような基準を定めて現在の良性石綿胸水の労災認定の礎となっている。この基準の妥当性を明らかにし、診断基準を明確にする調査研究が望まれる。

表 1

良性石綿胸水診断基準

①職業性石綿ばく露歴がある
②胸水穿刺により胸水の存在が確認されている
③下記の検査所見等により、胸水を来す他疾患を除外しうる
- 滲出性胸水
  - 胸水ADA値　50 IU/L未満
  - 胸水CEA値が血清正常値未満
- 胸水ヒアルロン酸値が 100,000 ng/ml未満
- 胸水細胞診　陰性
  - 胸水細胞分類で中皮細胞が５%未満
  - 胸部CTで悪性腫瘍が疑われるような胸膜病変を認めない
  - 胸腔鏡による胸膜病変の観察及び胸膜生検にて他疾患を否定できる
    ただし、胸腔鏡検査が出来ない症例では1年間経過観察で悪性腫瘍等他疾患を否定する

5．びまん性胸膜肥厚

1975 年の石綿吹付作業が禁止されて以降、石綿高濃度曝露がほとんどなくなった日本において、過去の曝露者に認められる良性胸膜病変としてびまん性胸膜肥厚が今後問題になると思われる。過去には良性石綿胸水からびまん性胸膜肥厚に至る症例が最も多いと報告されてきた。我々はびまん性胸膜肥厚と認定された症例のうち器質化胸水が残存する症例が 86%あることを報告した。そのため、良性石綿胸水とびまん性胸膜肥厚の線引きが必要となっている。これに対する試案として以下の基準を提唱している。

胸水を伴う症例をびまん性胸膜肥厚状態と診断するに際しては、CT 所見として器質化胸水にほぼ必須の、①胸水の不均一性と②胸水貯留部位における“ Crow's feet ” sign を含んで、③胸水中のエアー、④胸郭容量低下、⑤胸水量の固定化の５項目のうち３項目を満たしてスコア３点以上であれば、胸水が器質化を来し、そして固定化しびまん性胸膜肥厚状態と診断してよい病態とした。過去の労災あるいは救済における認定症例は全例スコア３点以上であるため妥当な基準とした。ただ、④の胸郭容量低下のみで、③胸水中のエアー、⑤胸水量の固定化が確認出来ない場合は、胸水貯留が慢性的な状況であることを一時点の CT 所見のみからは診断し難いため、一過性の胸水貯留に伴って生じる著しい呼吸機能障害を除外するために、少なくとも一度は経過を確認しておく必要があり、最短でも３ケ月は経過を観察したほうがよいと結論付けた[22]。

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Effects of repetitive transcranial magnetic stimulation on ER stress-related genes and glutamate, γ-aminobutyric acid and glycine transporter genes in mouse brain

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ABSTRACT

Repetitive transcranial magnetic stimulation (rTMS) is an emerging therapy for the treatment of psychiatric disorders. However, the mechanisms underlying the therapeutic effects of rTMS are still unclear, limiting its optimisation. Lasting effects suggest changes in disease-related genes, so we conducted gene chip and qRT-PCR analyses of genes associated with psychiatric diseases in the mouse brain at various times following 1, 20, 30 or 40 days of rTMS. Many genes were differentially expressed in the rTMS-treated mouse brain compared to sham controls, including genes encoding neurotransmitter transporters (upregulation of EAAT4, GLAST, GLT-1, GAT2, GAT4, GLYT1 and GLYT2), and endoplasmic reticulum (ER)-stress proteins (downregulation of IRE1α, IRE1β, and XBP1, upregulation of ATF6 and GRP78/Bip). Expression changes in many of these genes were also observed 10 days after the last rTMS treatment. In PC12 cells, rTMS upregulated GRP78/Bip mRNA and enhanced resistance against H_2O_2 stress. These results suggest that rTMS differentially modulates multiple genes associated with psychiatric and neurodegenerative disorders. Sustained changes in the expression of these genes may underlie the therapeutic efficacy of chronic rTMS.

1. Introduction

Repetitive transcranial magnetic stimulation (rTMS) is a novel non-invasive therapy for neurological and psychiatric diseases [1–4]. Since Barker et al. first demonstrated that it is possible to activate both peripheral nerves and brain tissue using external magnetic stimulation [1], TMS has gained acceptance as a pain-free and non-invasive diagnostic tool in neurology, such as for evaluating peripheral neuropathies [5].

In addition, several studies have reported therapeutic benefits of TMS for patients with psychiatric disorders, such as depression, Parkinson's disease and schizophrenia [6–8]. These psychiatric disorders are associated with dysfunction in monoaminergic and glutamatergic neurotransmitter systems, suggesting that the benefits of rTMS arise from modulation of these neurotransmitter signalling pathways. For example, deceased expression of glutamate and GABA transporter has been reported in the post mortal brain of Schizophrenia patients [9–11]. Based on the NMDAR (N-methyl-D-aspartate receptor) hypofunction hypothesis in schizophrenia, we speculated that rTMS might have effects on glutamatergic, GABAergic and glycinergic systems, including NMDAR, non-NMDAR, metabotropic GluR (glutamate receptor), glutamate transporter, GABA transporter, and glycine transporter. The glycine transporter is expressed in glia surrounding glutamatergic synapses and regulates synaptic glycine concentrations influencing NMDA receptor-mediated neurotransmission. Conversely, increased expression of GluR1 is found in the post mortal brain of Schizophrenia patients [12]. Because GluR1 is essential for the proliferation and growth of melanoma [13,14]; increased GluR1 might protect glutamatergic neurons. Because TMS is safe and relatively painlessness, it holds many possible applications as a therapeutic device for psychiatric disorders. However, the precise molecular mechanisms underlying the effects of TMS are unknown, which has impeded further optimisation for targeted regulation of processes involved in disease

Abbreviations: GLT-1, glial glutamate transporter-1; GLYT, glycine transporter; GLAST, glutamate/aspartate transporter; EAAC1, excitatory amino acid carrier 1; EAAT4, excitatory amino acid transporter 4; GABA, γ-aminobutyric acid; GAT, GABA transporter; ER, endoplasmic reticulum; GRP78/Bip, glucose-regulated protein 78/immunoglobulin heavy chain-binding protein; ATF6, activating transcription factor 6

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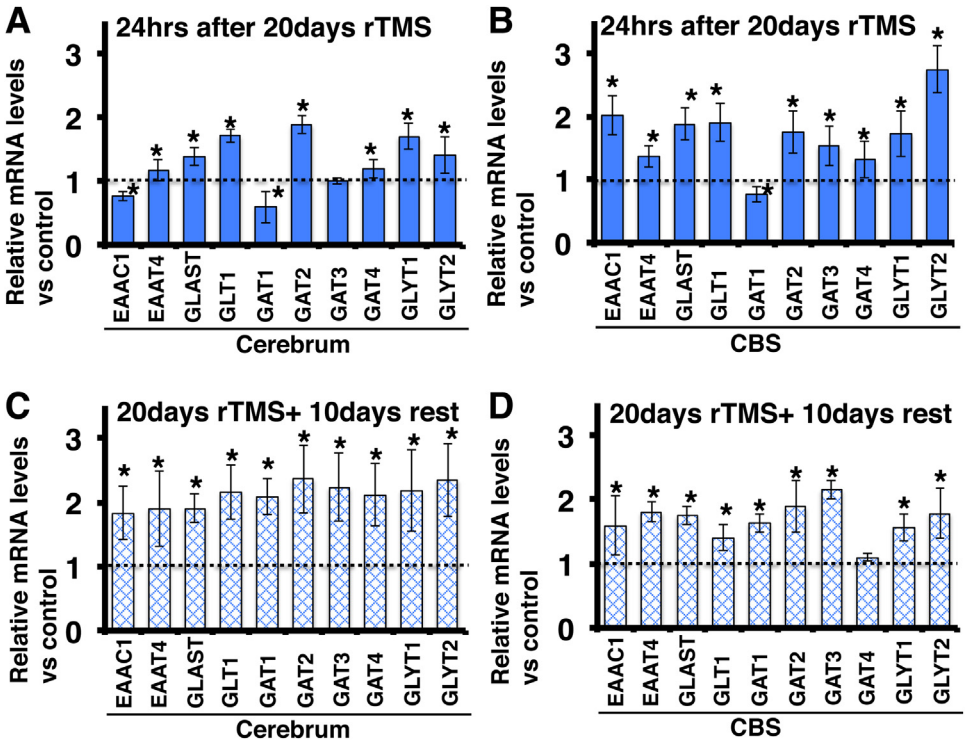

Fig. 1. Effects of chronic rTMS on glutamate, GABA and glycine transporter gene expression in mouse brain. The mRNA levels were determined at 24 h (A, B) and 10 days after 20 days of rTMS stimulation (C, D). (A, C) Effects of chronic rTMS on gene expression in the cerebrum. (B, D) Effects of chronic rTMS on gene expression in the cerebellum plus brain stem (CBS). All mRNA levels are normalised to GAPDH expression within the same sample. Values presented as mean ± SEM of five independent experiments, each performed in triplicate. *Significantly different from control at $P < 0.05$.

aetiology. Recent studies have demonstrated altered monoamine release after acute rTMS [15,16]. In addition, we reported changes in the expression levels of monoamine transporters, dopamine receptor 2, HSP70 and circadian rhythm-related genes after acute and chronic rTMS [17,18].

However, there have been few reports on changes in gene expression profiles following acute or chronic rTMS. This prompted us to evaluate gene expression changes in mouse brain following rTMS using gene chip technology. We demonstrate that rTMS induces lasting changes in the expression levels of multiple neurotransmitter transporter genes as well as several ER stress-related genes. Furthermore, we demonstrate that upregulation of the ER-stress gene GRP78/Bip in PC12 cells by rTMS enhances resistance against oxidative stress.

2. Materials and methods

2.1. Mice and rTMS conditions

Male C57Black mice (8 weeks old, 20–25 g) were chronically treated with rTMS for 20, 30 or 40 days (n = 50) or acutely for 1 day (n = 24). During treatment, the mice were housed in a light-controlled room (8:00 a.m. on, 8:00 p.m. off). A round coil (7.5 cm outer diameter) and a Nihon Kohden Rapid Rate Stimulator (Nihon Kohden, Japan) were used to perform the stimulation. For chronic rTMS, stimulation conditions were as follows: 20 Hz for 2 s, 20 times/day, inter-stimulus interval of 1 min and 30% machine output (representing about 0.75 T). The coil was placed over the head without touching the skull. Sham control mice were stimulated from a distance of more than 10 cm from the head. rTMS did not produce notable seizures or changes in behaviour, such as excessive struggling. Twenty-four hours after the last stimulation, the animals were sacrificed and their brains processed for further gene expression analysis. Mice subjected to acute rTMS (1 day using the same stimulus conditions) were sacrificed after 1, 4, 12 and 24 h for gene expression analysis. All the animal experiments were performed in compliance with institutional guidelines.

This study was approved by the Experimental Animal Committee of the RIKEN Institute and performed according to the guidelines for the care and use of experimental animals of RIKEN Institute (approval # H15-2B046).

2.2. RNA extraction

Whole mouse brain was divided at the midbrain into cerebrum and cerebellum with brain stem (CBS). Total RNA was isolated from cerebrum and CBS by acid–phenol extraction [19]. Poly(A)+ RNA was isolated from the samples using an mRNA purification kit (TaKaRa Bio, Japan) for expression analysis by TaqMan real-time RT-PCR. Primer Express Software (Applied Biosystems, Foster City, CA) was used to design the TaqMan primer and probe sets. Supplementary data 1 shows the nucleotide sequences of the primers. Contaminating genomic DNA was removed with RNase-free DNase I (TaKaRa Bio, Japan). Complementary DNAs were synthesised from 1 μg of mRNA per 100-μl reaction using MMLV Reverse Transcription Reagents (Invitrogen, Carlsbad, CA). The TaqMan PCR reaction mixture contained 15 μl of TaqMan Universal PCR Master Mix (Applied Biosystems) in a 30 μl reaction. Primers and probes were added in optimal concentrations. We used 1 μl of RT mix for each PCR. Each sample was amplified in duplicate and the experiment was repeated at least three times. PCR conditions were standard for the 7700 Sequence Detector System (Applied Biosystems): 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The mRNA quantity for each gene of interest was normalised to the quantity of GAPDH mRNA within each sample.

2.3. Cell culture

PC12 cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum (FCS) at 37 °C under 5% CO_2/95% air. Cells at subconfluence were harvested, diluted in culture medium and seeded in 24-well culture plates for cell viability assays or in 25 cm^2 culture flasks for total RNA extraction. The cells were cultured with or without daily rTMS for 15 days. Then, the cells in 24-well plates were treated with H_2O_2 for 2 h. Viability was evaluated by MTT assay [20].

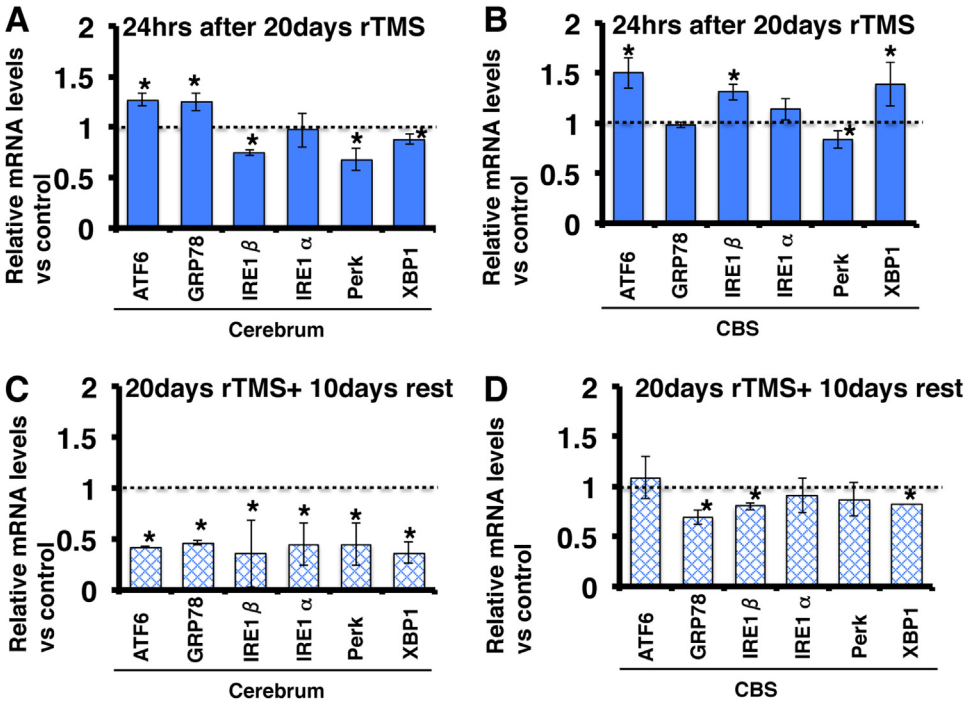

Fig. 2. Effects of rTMS on ER stress-related gene expression in mouse brain. The mRNA levels were determined at 24 h (A, B) and 10 days after 20 days of rTMS stimulation (C, D). (A, C) Effects of chronic rTMS on gene expression in the cerebrum. (B, D) Effects of chronic rTMS on gene expression in cerebellum plus brain stem (CBS). All mRNA levels are normalised to GAPDH expression in the same sample. Values presented as mean ± SEM of five independent experiments, each performed in triplicate. *Significantly different from control at $P < 0.05$.

Table 1
Summary of glutamate transporter gene expression changes following acute rTMS.

			1 h	4 h	12 h	24 h
EAAC1	Cerebrum	Control	1 ± 0.08	0.83 ± 0.06	1.04 ± 0.07	1.01 ± 0.13
		Mg+	1.04 ± 0.57	1.67 ± 0.27*	0.91 ± 0.06*	1.03 ± 0.08
	CBS	Control	1 ± 0.28	1.72 ± 0.35	1.79 ± 0.26	2.09 ± 0.3
		Mg+	0.93 ± 0.03	1.73 ± 1.28	1.69 ± 0.61	1.61 ± 0.28
EAAT4	Cerebrum	Control	0.99 ± 0.13	0.82 ± 0.07	1.05 ± 0.09	0.99 ± 0.1
		Mg+	1.01 ± 0.13	1.7 ± 0.29*	0.88 ± 0.07*	1.05 ± 0.08
	CBS	Control	1 ± 0.31	1.53 ± 0.38	1.74 ± 0.46	1.89 ± 0.15
		Mg+	1.06 ± 0.05	1.56 ± 0.85	1.62 ± 0.7*	1.57 ± 0.3
GLAST	Cerebrum	Control	1 ± 0.14	0.79 ± 0.05	0.97 ± 0.05	0.98 ± 0.14
		Mg+	0.99 ± 0.12	1.64 ± 0.33*	0.87 ± 0.05*	0.99 ± 0.07
	CBS	Control	1 ± 0.02	1.6 ± 0.07	1.49 ± 0.29	1.98 ± 0.31
		Mg+	0.81 ± 0.04*	1.15 ± 0.09*	1.38 ± 0.14	1.45 ± 0.05*
GLT1	Cerebrum	Control	1 ± 0.17	0.73 ± 0.04	0.86 ± 0.05	0.93 ± 0.11
		Mg+	0.96 ± 0.11	1.45 ± 0.31*	0.76 ± 0.04*	0.88 ± 0.09
	CBS	Control	1 ± 0.34	1.51 ± 0.32	1.38 ± 0.33	2.39 ± 0.54
		Mg+	0.76 ± 0.05	1.34 ± 0.9*	1.56 ± 0.22*	1.36 ± 0.26*
GAT1	Cerebrum	Control	1 ± 0.06	0.8 ± 0.05	0.97 ± 0.05	0.91 ± 0.11
		Mg+	0.99 ± 0.13	1.64 ± 0.32*	0.83 ± 0.04*	0.95 ± 0.08
	CBS	Control	1 ± 0.38	1.78 ± 0.47	1.85 ± 0.45	1.97 ± 0.32
		Mg+	0.92 ± 0.06	1.76 ± 1.05	1.73 ± 0.5	1.69 ± 0.41
GAT2	Cerebrum	Control	0.99 ± 0.1	0.79 ± 0.07	0.95 ± 0.07	0.99 ± 0.19
		Mg+	0.87 ± 0.04*	1.52 ± 0.3*	0.84 ± 0.04*	0.89 ± 0.04
	CBS	Control	1 ± 0.16	1.8 ± 0.14	1.82 ± 0.16	1.81 ± 0.09
		Mg+	0.84 ± 0.03*	2.11 ± 1.37*	1.55 ± 0.06*	1.51 ± 0.6*

Values are mean ± SEM of three independent experiments, each performed in triplicate.
* Significantly different from control at $P < 0.05$.

2.4. Data analysis

The data are presented as mean ± SE of at least three independent experiments, each performed in triplicate or duplicate. Means were compared by ANOVA (Figs. 1 and 2) or Student's *t*-test as appropriate.

3. Results

3.1. Gene expression changes in mouse brain following rTMS

We stimulated the brains of 8-week-old C57 Black mice for 20, 30 or 40 days by rTMS and analysed the changes in gene expression using the Affymetrix GeneChip microarray. GeneChip analysis revealed altered expression levels of multiple mRNAs, including those encoding glutamate and glycine transporters as well as ER stress-related genes, in both cerebrum and CBS [21,22]. In order to quantify these changes, we then measured mRNA levels by qRT-PCR (Figs. 1 and 2, Tables 1–3).

3.2. Effects of acute and chronic rTMS on transporter genes in mouse brain

The GeneChip data showed that glutamate and glycine transporter mRNAs were altered after chronic rTMS. Thus, we examined the mRNA

Table 2
Summary of GABA and glycine transporter gene expression changes following rTMS.

			1 h	4 h	12 h	24 h
GAT3	Cerebrum	Control	0.99 ± 0.15	0.8 ± 0.07	0.94 ± 0.07	0.95 ± 0.15
		Mg+	0.89 ± 0.07	1.6 ± 0.31*	0.88 ± 0.05	0.92 ± 0.08
	CBS	Control	0.99 ± 0.02	1.47 ± 0.22	1.47 ± 0.35	1.55 ± 0.05
		Mg+	1.12 ± 0.19	1.61 ± 0.98*	1.18 ± 0.29	1.33 ± 0.24*
GAT4	Cerebrum	Control	0.99 ± 0.06	0.8 ± 0.03	0.99 ± 0.04	0.96 ± 0.11
		Mg+	0.99 ± 0.1	1.69 ± 0.32*	0.85 ± 0.04*	0.97 ± 0.08
	CBS	Control	1 ± 0.04	2.12 ± 0.22	1.92 ± 0.29	2.42 ± 0.39
		Mg+	1.32 ± 0.07*	1.5 ± 0.46*	2.14 ± 0.62	2.16 ± 0.47
GLYT1	Cerebrum	Control	1 ± 0.12	0.81 ± 0.05	1.02 ± 0.06	1.04 ± 0.18
		Mg+	0.94 ± 0.12	1.6 ± 0.21*	0.85 ± 0.04*	1.03 ± 0.1
	CBS	Control	1 ± 0.32	1.65 ± 0.42	1.63 ± 0.36	1.77 ± 0.21
		Mg+	0.96 ± 0.03	1.55 ± 0.96	1.77 ± 0.44	1.74 ± 0.46
GLYT2	Cerebrum	Control	1 ± 0.09	0.72 ± 0.07	1.02 ± 0.02	0.97 ± 0.49
		Mg+	0.87 ± 0.11*	1.57 ± 0.17*	0.77 ± 0.02*	1.28 ± 0.4
	CBS	Control	0.52 ± 0.3	1 ± 0.14	1.02 ± 0.19	1.18 ± 0.16
		Mg+	0.37 ± 0.03	1.12 ± 0.79	1.07 ± 0.43	0.82 ± 0.16*

Values are mean ± SEM of three independent experiments, each performed in triplicate.
* Significantly different from control at $P < 0.05$.

Table 3
Summary of ER stress-related gene expression changes following acute rTMS.

			1 h	4 h	12 h	24 h
ATF6	Cerebrum	Control	1 ± 0.01	1.04 ± 0.05	0.86 ± 0.06	1.24 ± 0.15
		Mg+	0.89 ± 0.08*	0.87 ± 0.14*	0.88 ± 0.05	0.8 ± 0*
	CBS	Control	1 ± 0.05	0.87 ± 0.07	1.02 ± 0.09	1.06 ± 0.17
		Mg+	0.52 ± 0.11*	1.05 ± 0.25	0.8 ± 0.01*	0.41 ± 0.61*
GRP78/Bip	Cerebrum	Control	1 ± 0.15	1.42 ± 0.31	0.81 ± 0.02	1.19 ± 0.19
		Mg+	1.38 ± 0.19*	0.86 ± 0.08*	1.14 ± 0.13*	0.76 ± 0.05*
	CBS	Control	1 ± 0.02	0.95 ± 0.04	1.16 ± 0.13	2.19 ± 0.15
		Mg+	1.4 ± 0.29*	1.06 ± 0.05	0.74 ± 0.04*	1.49 ± 0.45*
IRE1β	Cerebrum	Control	1 ± 0.1	1.57 ± 0.18	1.27 ± 0.07	1.34 ± 0.05
		Mg+	1.03 ± 0.12	1.21 ± 0.16*	0.96 ± 0.1*	1.2 ± 0.08
	CBS	Control	1 ± 0.18	1.39 ± 0.25	0.97 ± 0.44	1 ± 0.1
		Mg+	1.15 ± 0.14*	1.63 ± 0.02*	0.99 ± 0.09*	1.16 ± 0.13*
IRE1α	Cerebrum	Control	1 ± 0.04	1.43 ± 0.07	1.05 ± 0.06	1.22 ± 0.09
		Mg+	0.86 ± 0.05*	1.04 ± 0.19*	1.02 ± 0.03	0.92 ± 0.04*
	CBS	Control	1 ± 0.11	1.04 ± 0.12	1 ± 0.12	1.34 ± 0.07
		Mg+	0.56 ± 0.12*	1.05 ± 0.25	1 ± 0.09	1.37 ± 0.04
PERK	Cerebrum	Control	1 ± 0.09	1.12 ± 0.16	0.91 ± 0.04	1.04 ± 0.1
		Mg+	0.82 ± 0*	1.07 ± 0.11	0.91 ± 0.06	1.03 ± 0.05
	CBS	Control	1 ± 0.11	0.92 ± 0.12	0.95 ± 0.12	1.05 ± 0.07
		Mg+	0.79 ± 0.12*	0.84 ± 0.25	0.73 ± 0.09*	1.46 ± 0.04*
XBP1	Cerebrum	Control	1 ± 0.1	1 ± 0.03	0.83 ± 0.04	1 ± 0.02
		Mg+	1.14 ± 0.03*	0.85 ± 0.09*	0.97 ± 0.06*	0.77 ± 0.05*
	CBS	Control	1.01 ± 0.01	1.08 ± 0.02	0.94 ± 0.01	1.94 ± 0.11
		Mg+	1.3 ± 0.11*	1.26 ± 0.01*	0.79 ± 0.09*	1.24 ± 0.46*

Values are mean ± SEM of three independent experiments, each performed in triplicate.
* Significantly different from control at $P < 0.05$.

levels of the glutamate transporters EAAC1, EAAT4, GLAST and GLT1, the GABA transporters GAT1–4, and the glycine transporters GLYT1 and 2 both 24 h and 10 days after 20 days of rTMS. Twenty-four hours after the last rTMS application, EAAT4, GLAST, GLT1, GAT2, GAT4, GLYT1 and GLYT2 mRNA levels were upregulated in the cerebrum (Fig. 1A), and EAAC1, EAAT4, GLAST, GLT1, GAT2, GAT3, GAT4, GLYT1 and GLYT2 mRNA levels were upregulated in the CBS (Fig. 1B). Conversely, EAAC1 and GAT1 mRNA levels were downregulated in the cerebrum (Fig. 1A), while GAT1 mRNA level was downregulated in the CBS (Fig. 1B). Ten days after the last rTMS treatment, EAAC1, EAAT4, GLAST, GLT1, GAT1, GAT2, GAT3, GAT4, GLYT1 and GLYT2 mRNA levels were upregulated in both cerebrum and CBS (Fig. 1C, D), while GAT4 mRNA level was upregulated only in the cerebrum (Fig. 1D).

We next examined the acute effects of a single days' rTMS administration after 1, 4, 12 and 24 h. These measurements revealed temporally complex and region-specific expression changes in transporter and ER stress-related genes. Expression changes in glutamate transporter genes are summarised in Table 1, GABA and glycine transporter genes in Table 2, and ER-stress genes in Table 3. After acute rTMS, EAAC1, GAT1 and GLYT1 mRNA levels were upregulated at 4 h and downregulated at 12 h in the cerebrum (Tables 1 and 2). EAAT4 mRNA levels were upregulated at 4 h and downregulated at 12 h in the cerebrum, while expression levels were unchanged in CBS at 4 h before decreasing at 12 h (Table 1). GLAST mRNA levels were upregulated at 4 h and downregulated at 12 h in the cerebrum. In contrast, expression was downregulated after 1, 4 and 24 h in CBS (Table 1). GAT2 mRNA levels were upregulated at 4 h and downregulated at 1 and 12 h in the cerebrum, but upregulated at 4 h and downregulated after 1, 4 and 24 h in CBS (Table 1). GAT3 mRNA levels were upregulated at 4 h in the cerebrum and CBS, but then downregulated only in the CBS at 24 h (Table 2). GAT4 mRNA levels were upregulated at 4 h and downregulated at 12 h in the cerebrum, but upregulated at 1 h and downregulated at 12 h in CBS (Table 2). GLYT2 mRNA levels were upregulated at 4 h and downregulated at 1 and 12 h in the cerebrum, but

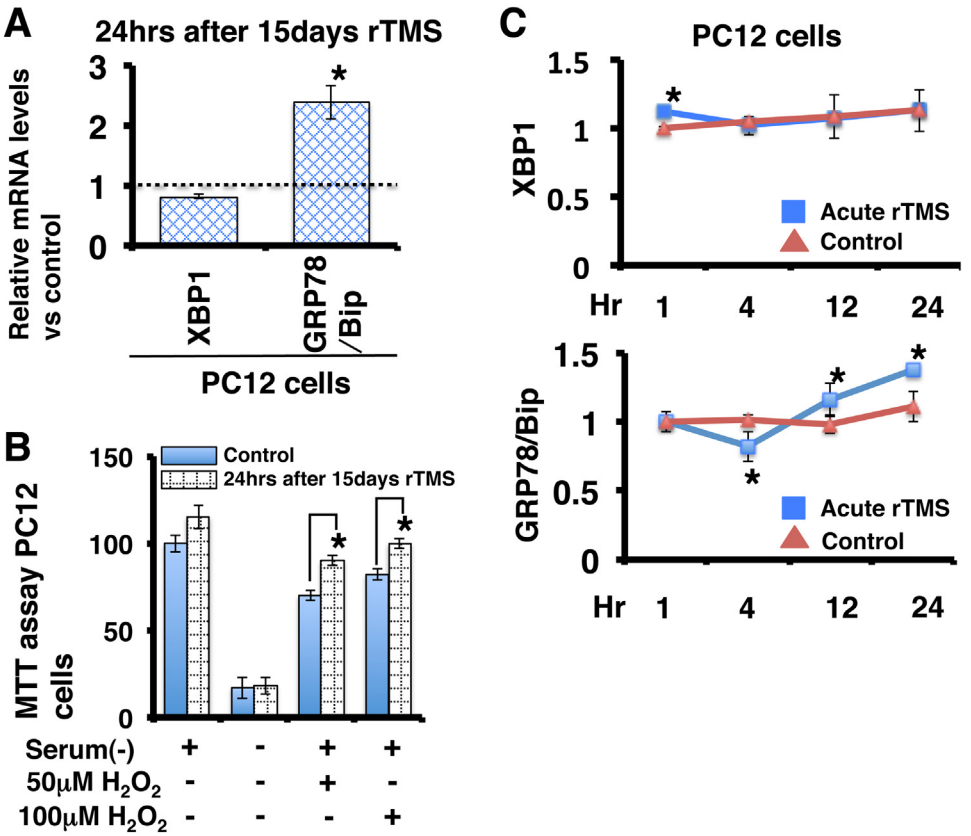

Fig. 3. Effects of chronic and acute rTMS on XBP1 and GRP78/Bip mRNA expression levels and H_2O_2 resistance in P12 cells. (A) Effects of chronic rTMS on XBP1 and GRP78/Bip mRNA levels in PC12 cells. (B) Viability of rTMS-treated and control PC12 cells exposed to H_2O_2 for 2 h. (C) Effects of acute rTMS on XBP1 and GRP78/Bip mRNA levels in PC12 cells at 1, 4, 12 and 24 h after stimulation. Values presented as mean ± SEM of three independent experiments, each performed in triplicate. *Significantly different from control at $P < 0.05$.

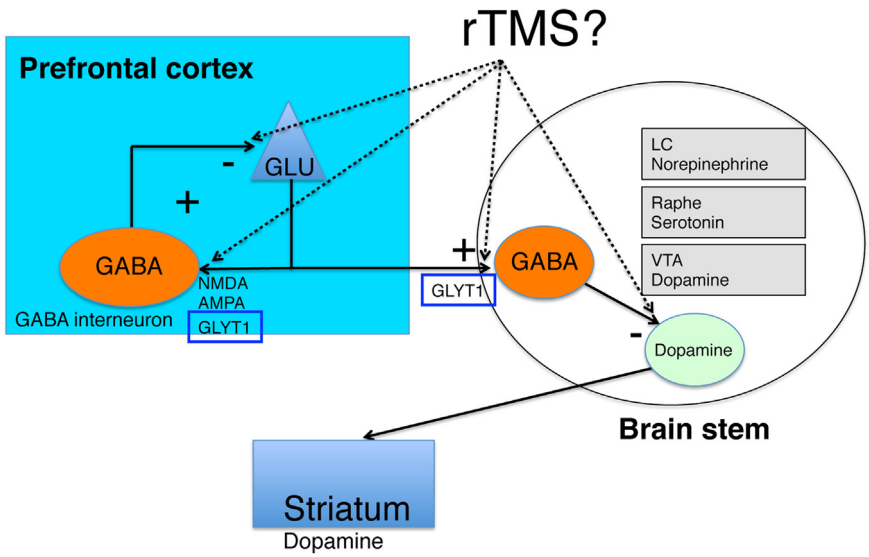

Fig. 4. Model for differential regulation of neurotransmitter transporters in mouse brain by rTMS. Chronic rTMS may alter glutamatergic, GABAergic and glycinergic neurotransmission by changing the rate of transmitter uptake from the synaptic cleft.

downregulated at 24 h in CBS (Table 2). In summary, rTMS appears to differentially regulate neurotransmitter transporter genes both dynamically following acute treatment and in a more sustained manner following chronic treatment.

3.3. Effects of acute and chronic rTMS on ER stress-related genes in mouse brain

GeneChip data revealed that multiple ER stress-related genes were also altered by rTMS [22], so examined the effects of acute and chronic rTMS on specific ER stress-related genes by qRT-PCR. Twenty-four hours after 20 days of rTMS, ATF6 and GRP78/Bip mRNA levels were upregulated in the cerebrum (Fig. 2A), and ATF6, IRE1β and XBP1 mRNA levels were upregulated in CBS (Fig. 2B). However, IRE1β, PERK and XBP1 mRNA levels were downregulated in the cerebrum (Fig. 2A) and PERK mRNA level was downregulated in CBS (Fig. 2B). Ten days after the last rTMS treatment, ATF6, GRP78/Bip, IRE1α, IRE1β, PERK and XBP1 mRNA levels were downregulated in the cerebrum (Fig. 2C), and GRP78/Bip, IRE1 β, PERK and XBP1 mRNA levels were downregulated in CBS (Fig. 2D). There were no changes in ATF6, IRE1α and PERK mRNA levels in CBS (Fig. 2D).

We then examined the acute effects of rTMS at 1, 4, 12 and 24 h

(Table 3). ATF6 mRNA level was downregulated in the cerebrum at 1, 4 and 24 h, and in CBS at 1, 12 and 24 h after acute rTMS. GRP78/Bip mRNA level was upregulated at 1 and 12 h in the cerebrum and at 1 h in CBS, but was downregulated at 4 and 24 h in the cerebrum and at 12 and 24 h in CBS. IRE1β mRNA level was upregulated at 1, 4 and 24 h and downregulated at 12 h in CBS, but was downregulated at 4 and 12 h in the cerebrum. IRE1α mRNA level was downregulated at 1 h in the CBS, and downregulated at 1, 4 and 12 h in the cerebrum. PERK mRNA level was downregulated at 1 and 12 h in CBS and at 1 h in the cerebrum, but upregulated at 24 h in the CBS. XBP1 mRNA level was downregulated at 12 and 24 h in CBS and at 4 h in the cerebrum, but upregulated after 1 and 12 h in the cerebrum and at 1 and 4 h in CBS. Thus, similar to neurotransmitter transporter genes, rTMS induced temporally complex and region-specific expression changes in ER stress-related genes.

3.4. Effects of acute and chronic rTMS on ER stress-related genes in PC12 cells

The effects of rTMS on ER stress-related genes were also examined in PC12 cells to investigate the functional significance. GRP78/Bip mRNA expression was upregulated after 15 days of rTMS in PC12 cells (Fig. 3A). After acute rTMS, XBP1 mRNA level was upregulated at 1 h, while GRP78/Bip mRNA level was downregulated at 4 h and upregulated at 12 and 24 h (Fig. 3C). ER stress-related genes are known to be induced by H_2O_2 via glutamate and glycine stimulation as a compensatory response to prevent cytotoxic protein misfolding, so we investigated whether rTMS increases PC12 cell resistance against H_2O_2. Indeed, 15 days of rTMS increased cell viability following 2 h of H_2O_2 exposure compared to controls (Fig. 3B).

4. Discussion

Repetitive TMS is a promising treatment for psychiatric disorders. Persistent therapeutic effects suggest that rTMS induces lasting changes in the expression of genes involved in these disorders, such as genes associated with stress-responses and neurotransmission. To study such effects, we measured gene expression changes by gene chip arrays and qRT-PCR in mouse brain following an acute (1-day) or chronic (20-day) rTMS protocol. We have previously shown that acute and chronic rTMS alters monoamine transporter (MAT) mRNA expression, protein levels and function [17], dopamine receptor 2 mRNA expression, protein levels and function, circadian rhythm-related gene expression and both mRNA and protein expression levels of the stress response gene HSP70 [18]. Here we confirmed differential modulation of transporter and ER stress-related genes reported in previous studies [21,22] and provide evidence for enhanced neuroprotection following rTMS.

In addition to changes in mouse cerebrum, we found that GRP78/Bip mRNA level was upregulated in PC12 cells by acute and chronic rTMS. Furthermore, 15 days of rTMS increased the resistance of PC12 cells against H_2O_2 toxicity. These results indicate that rTMS may also slow the progression of neurodegenerative disorder, such as Huntington's disease, through GRP78/Bip induction. Further investigations are needed on the effects of chronic rTMS because acute rTMS increased GRP78/Bip mRNA expression in the cerebrum 24 h after 20 days of rTMS, whereas other stress-associated genes such as ATF6, IRE1β, PERK and XBP1 were downregulated. Thus, the ER-stress response induced by rTMS appears highly specific.

Expression levels of EAAT4, GLAST, GLT1, GAT2, GAT4, GLYT1 and GLYT2 were upregulated in the cerebrum after chronic rTMS (Fig. 1A), while these same genes plus EAAC1 and GAT3 were upregulated in CBS (Fig. 1B). Furthermore, EAAC1, EAAT4, GLAST, GLT1, GAT1, GAT2, GAT3, GAT4, GLYT1 and GLYT2 mRNA levels were still upregulated in the cerebrum and CBS 10 days after the last rTMS administration (Fig. 1C, D). Thus, rTMS appears to induce widespread and sustained increases in glutamate, GABA and glycine transporters, possibly resulting in altered transmitter signalling. Indeed, the efficacy of NMDA receptor antagonists such as memantine for the treatment of dementia, obsessive-compulsive disorder (OCD), and certain schizophrenia symptoms suggests that chronic rTMS may result in symptom improvement by upregulating transporter-mediated glutamate uptake. Our results also suggest that ER stress-related genes may be involved in glutamate and glycine transporter expression changes induced by chronic rTMS.

In conclusion, the therapeutic effects of chronic rTMS may depend on modulation of ER stress-related genes and glutamate, glycine and GABA transporter genes (Fig. 4). Further research is needed to identify the region-specific functional changes resulting from up- and down-regulation of these genes following rTMS. Such information could facilitate the development of more effective rTMS protocols with fewer side effects.

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Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbrep.2018.10.015.

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Blood Cancer Journal

ARTICLE Open Access

CD26 is a potential therapeutic target by humanized monoclonal antibody for the treatment of multiple myeloma

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Abstract

CD26, a 110-kDa transmembrane glycoprotein that is expressed on several tumor cells including malignant lymphoma, has been implicated in tumorigenesis: however, little is known regarding its role in multiple myeloma (MM). Recently, we identified CD26 expression on human osteoclasts (OCs) and demonstrated that humanized IgG_1 monoclonal antibody targeting CD26, huCD26mAb, inhibits human OC differentiation. Herein, we show that CD26 expression was present on plasma cells in the bone marrow tissues of MM patients. In vitro immunostaining studies revealed that although CD26 expression was low or absent on MM cell lines cultured alone, it was intensely and uniformly expressed on MM cell lines co-cultured with OCs. The augmented CD26 expression in MM cells was exploited to enhance anti-MM efficacy of huCD26mAb via a substantial increase in antibody-dependent cytotoxicity (ADCC) but not complement-dependent cytotoxicity (CDC). Moreover, huCD26mAb in combination with novel agents synergistically enhanced huCD26mAb induced ADCC activity against CD26+ MM cells compared with each agent alone. huCD26mAb additionally reduced the ratio of the side population (SP) fraction in CD26+ MM cells by ADCC. Finally, huCD26mAb significantly reduced the MM tumor burden and OC formation in vivo. These results suggest that CD26 is a potential target molecule in MM and that huCD26mAb could act as a therapeutic agent.

Introduction

Despite remarkable advances in the current treatment options, including proteasome inhibitors (PIs) and immunomodulatory drugs (IMiDs) as well as high-dose chemotherapy followed by autologous stem cell transplantation, which have significantly improved the overall survival (OS) of multiple myeloma (MM) patients, most of them relapse or ultimately become refractory due to the residual disease within the MM microenvironment[1,2]. Therefore, the development of alternative therapeutic approaches, based on the understanding of the biology of the disease, is urgently required. Recently, a new generation of novel agents including PIs (carfilzomib and ixazomib)[3–5], IMiDs (pomalidomide)[6,7], and histone deacetylase inhibitors (HDACi: panobinostat)[8] have emerged and are expected to further improve the clinical outcome of MM patients.

The use of immunotherapy in the treatment of cancers has been accelerating and increasing evidence has shown that antibody therapies can improve the outcome of patients with cancer[9,10]. Rituximab, a chimeric murine/human anti-CD20 monoclonal $IgG_1\kappa$ antibody targeting B cells, is currently indicated for the treatment of B-cell non-Hodgkin's lymphoma (NHL) and chronic lymphocytic leukemia (CLL) and exerts significant activity, especially in combination with cytotoxic chemotherapy[9]. In contrast, clinical trials of rituximab therapy in MM have been disappointing, showing that few patients with MM achieve only minimal responses[10] because only a small number of patients express CD20 in plasma cells[11,12]. Immunotherapeutic approaches for MM have been long awaited because of the significantly impaired

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immune system due to the inhibition of normal plasma cells and the multiple mechanisms of immune evasion by MM cells, including the lack of unique targets that are highly expressed in MM cells but not normal cells, the enhanced expression of inhibitory ligands, such as programmed cell death ligand 1 (PDL1), and the recruitment of regulatory T cells (Tregs). Recently, novel efficacious mAbs have been developed based on the identification of target antigens, such as elotuzumab, a humanized IgG_1 monoclonal antibody targeting signaling lymphocyte activation molecule family member 7 (SLAMF7, CS1)[13] and daratumumab, a humanized $IgG_1\kappa$ monoclonal antibody directed against CD38[14]. These novel mAbs are effective for the treatment of MM patients who have received >3 prior lines of therapy or who were double refractory to a PI and an IMiD. These mAbs have become increasingly used in combination with bortezomib (BTZ)/dexamethasone (Dexa) or lenalidomide (Lena)/Dexa. These combinations have been shown to significantly improve overall response rates (ORR) and progression-free survival (PFS) in patients with MM compared with these agents alone[15–22].

CD26, a 110-kDa transmembrane glycoprotein with DPPIV activity, is widely expressed in a various normal cells, including T lymphocytes, natural killer (NK) cells, endothelial cells, and epithelial cells[23–26]. Additionally, CD26 is expressed in several tumor cells and is involved in T-cell activation and tumorigenesis (Fig. 1a)[23–28]: however, its role in plasma cell malignancies has not been characterized yet. We recently identified that CD26 is intensely expressed in human osteoclasts (OCs) in osteolytic bone tumors, including MM, and that huCD26mAb, a humanized IgG_1 monoclonal antibody that directly targets CD26, inhibits human OC differentiation[29]. In addition, we detected that CD26 is expressed on MM cells in the bone marrow (BM) tissues of MM patient. In the present study, we show that CD26 was intensely and uniformly expressed in MM cell lines co-cultured with OCs, while its expression was low or absent in those cultured alone in vitro. We further clarify CD26 as a potential target for the treatment of MM. We herein examine the therapeutic impact of novel huCD26mAb on MM cell growth, cell death via antibody-dependent cellular cytotoxicity (ADCC) and its associated osteolytic bone disease in vitro and in vivo and validate that huCD26mAb could be a promising immunotherapeutic option for MM.

Materials and methods

Cell lines

The cancer cell lines, U266, KMM1, KMS11, KMS12, KMS18, KMS20, KMS21, KMS34, and IM9, were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), KMS26, KMS27, KMS28, and RPMI8226 were obtained from the National Institute of Biomedical Innovation, Health and Nutrition (NIBIOHN, Osaka, Japan). Nakadai was kindly gifted by Dr Hattori's laboratory. These cell lines were maintained in RPMI-1640 medium (Invitrogen, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Grand Island, NY, USA), penicillin and streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

Reagents and cells

Human bone marrow mononuclear cells (BM-MNCs) derived from MM patients and bone marrow stromal cells (BMSCs) were purchased from Lonza (Walkersville, MD, USA). Human NK cells were obtained from Biotherapy Institute of Japan (Tokyo, Japan). Recombinant human macrophage colony stimulating factor (M-CSF), soluble receptor activator of nuclear factor κ B ligand (sRANKL), Interleukin-6 (IL-6), stromal cell-derived factor (SDF)-1, tumor necrosis factor alpha (TNFα), B-cell activating factor of TNF family (BAFF), and a proliferation-inducing ligand (APRIL) were obtained from Peprotec (Rockyhill, NJ, USA). Levlimid® (lenalidomide: Celgene, Summit, NJ, USA), Velcade® (bortezomib: Millennium, Cambridge, MA, USA), and Decadron® (dexamethasone: Sigma Aldrich, St. Louis, MO, USA) were used as therapeutic agents. huCD26mAb, humanized IgG_1, was generously provided by Y's Therapeutics (Fig. 1a) (Tokyo, Japan). The huCD26mAb employed in the present study was generated by utilizing the complementarity-determining regions of the murine anti-human CD26mAb, 14D10, previously developed in our laboratory[30,31], which has no cross-reactivity to murine CD26. Isotype IgG_1 (Sigma Aldrich) was used as a control.

Co-culture of MM cells and OCs

Human BM-MNCs (5×10^5 cells per well) were cultured with human M-CSF (25 ng/ml; from day 0) plus sRANKL (50 ng/ml; from day 3) for 5 to 7 days in α-minimum essential medium (α-MEM) (Life Technologies) supplemented with 10% FBS, penicillin and streptomycin in type 1 collagen-coated 24-well plates (OC culture), described previously[29]. After washing the OCs with phosphate-buffered saline (PBS) three times to detach and remove any non-adherent cells, a panel of MM cells was co-cultured with OCs in 24-well plates (1 ml per well) in triplicate for 72 h or cultured alone in the presence or absence of the indicated concentrations of huCD26mAb[32–34]. In addition, MM cells were placed in 0.45 μm pore size transwell inserts (Corning, Corning, NY, USA) in wells containing OCs (co-culture without direct contact). At the end of each experiment, the MM cells were collected, counted with trypan blue staining and subjected to each assay. Details of the bone resorption

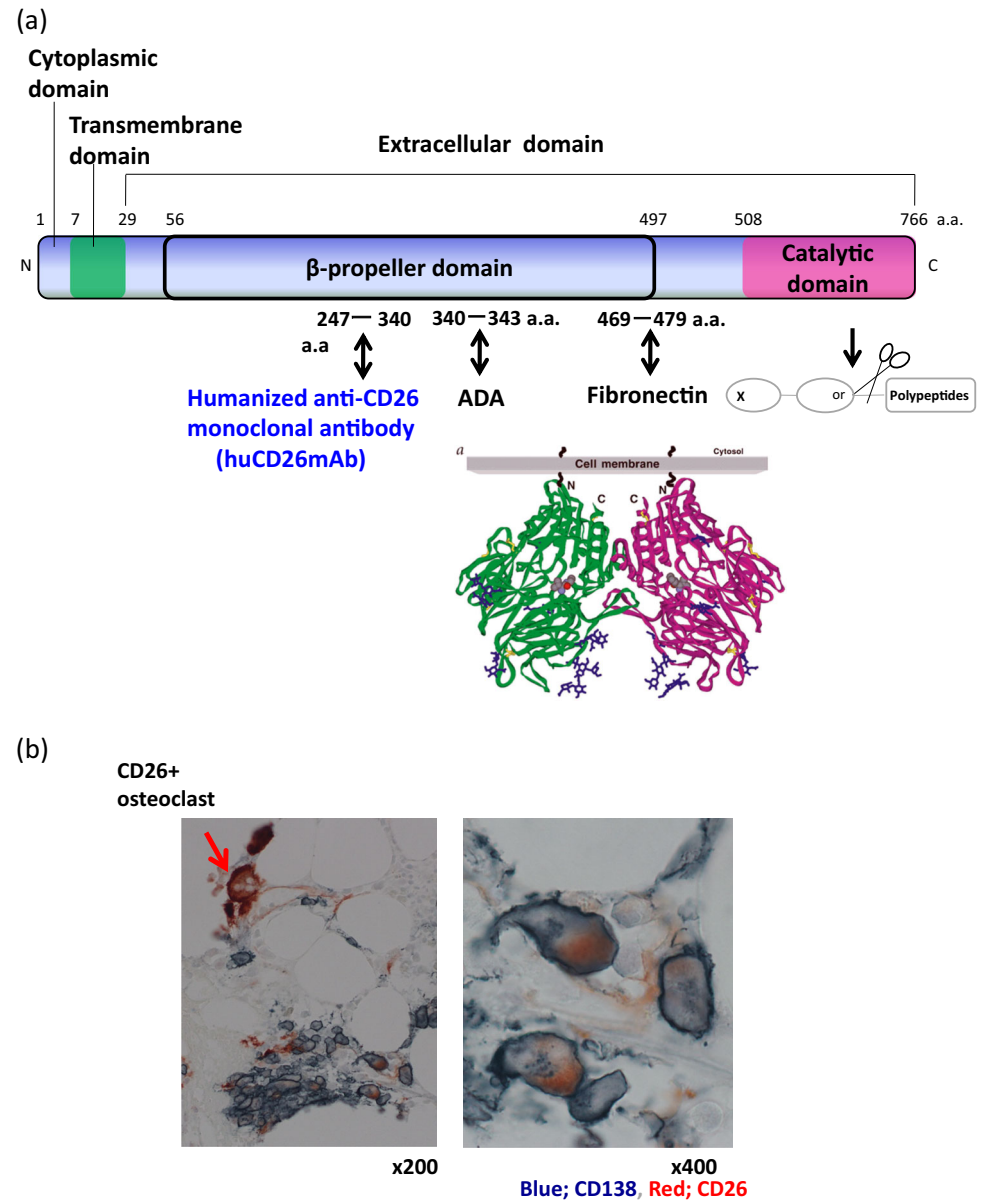

Fig. 1 CD26 expression in plasma cells of bone marrow tissues from multiple myeloma (MM) patient. a CD26 is composed of a short cytoplasmic domain, a transmembrane region and an extracellular domain with DPPIV activity. The epitope recognized by huCD26mAb is located at the 247th to 340th amino acid region of CD26. **b** Bone marrow tissues of MM patient revealed that CD26/CD138-double positive plasma cells exhibited CD138 staining with a membranous as well as a cytoplasmic expression pattern, and CD26 exhibited a cytoplasmic expression pattern. Original magnification; ×200 (upper panel), ×400 (lower panel). OCs in bone marrow tissue derived from MM patient were also positive for CD26

assay are described in the Supplementary Materials and Methods.

Immunohistochemistry and enzyme-histochemistry

Bone marrow biopsy samples were fixed in 10% neutral-buffered formalin and embedded in paraffin. Paraffin-embedded tissue sections were deparaffinized and hydrated. Further details are provided in the Supplementary Materials and Methods.

Antibody-dependent cellular cytotoxicity (ADCC) assay

The effect of huCD26mAb to induce human NK effector cell-dependent lysis of MM cells was evaluated by calcein-AM release assay. Further details are provided in the Supplementary Materials and Methods.

In vivo anti-MM therapy with huCD26mAb

To assess the effect of huCD26mAb against tumorigenicity in MM in vivo, 2 xenograft models were

established either by subcutaneous inoculation of MM cells (NOD/SCID MM model) or direct intra-bone injection of MM cells into human bone grafts, subcutaneously implanted in 6-week female NOD/SCID mice, which were housed in our animal research facility (NOD/SCID-hu model)[35–37]. Further details are provided in the Supplementary Materials and Methods.

Statistical analysis

All statistical analyses were performed using Student's *t*-test for two group comparisons and p-values <0.05 were considered statistically significant. The data are presented as the mean values with 95% confidence intervals, and the results are representative of three independent experiments.

Study approval

Experimental procedures and study protocols were approved by the Ethics Committee of Keio University School of Medicine (permission ID number 2013-0034). Informed consent was obtained from all patients. All studies of human subjects were performed according to the principles outlined in the Declaration of Helsinki.

Results

huCD26mAb inhibits OC differentiation derived from bone marrow mononuclear cells (BM-MNCs) from MM patients

Following the 7-day culture of M-CSF/sRANKL-stimulated BMNCs derived from MM patients in type 1 collagen-coated 24-well plates (OC culture), a large number of multinuclear OCs formed that were positive for tartrate-resistant acid phosphatase (TRAP) and CD26 (Figure S1a). huCD26mAb induced a significant reduction in TRAP+ multinuclear mature OC numbers (>3 nuclei) in a dose-dependent manner (Figure S1b, c). Subsequently, it reduced bone resorptive activity in OCs derived from MM patients in a dose-dependent manner (Figure S1d).

CD26 is intensely expressed in MM cells, co-cultured with OCs

BM tissues of MM patient revealed that the CD26 +/CD138+ plasma cells stained with CD138 with a membranous as well as a cytoplasmic expression pattern and CD26 with a cytoplasmic expression pattern (Fig. 1b). It has been reported that cellular components within the BM microenvironment, including OCs or BMSCs, enhance MM cell proliferation and survival by cell-to-cell contact between MM cells and the BM microenvironment. To further elucidate the role of CD26 in MM cells and the consequences of the interaction between MM cells and OCs, we established a MM cell-OC co-culture system. Initially, we examined CD26 expression in 14 MM cell lines (U266, KMM1, Nakadai, KMS11, KMS12, KMS18, RPMI8226, KMS20, KMS21, KMS26, KMS27, KMS28, KMS34, and IM9). Flow cytometry analysis showed that CD26 was barely or not detected in MM cell lines cultured alone (mono-culture) (Fig. 2a), but its expression increased in cell lines co-cultured with OCs (Fig. 2b). Immunostaining revealed that OCs express high level of CD26 compared with tested MM cell lines (U266, KMS18, KMS26, KMS27, and KMS28) cultured alone, which had low or slightly detectable level of CD26 (Fig. 2c). After co-culture, CD26 expression remained high in OCs and was upregulated in MM cell lines (Fig. 2c). Moreover, when subsequently cultured alone after removal of co-culture with OCs, CD26 expression in MM cell lines was downregulated again (Fig. 2c). In addition, CD26 protein expression was observed in MM cell lines co-cultured with OCs, while it was low or absent in those cultured alone according to immunoblot analysis (Fig. 2d). Further, to determine whether CD26 may be a potential biomarker for MM, we used an enzyme-linked immunosorbent assay (ELISA) to investigate whether there were detectable levels of CD26/DPPIV in the supernatant of MM cells or OCs grown in mono-culture and in that of the co-culture of MM cells with OCs. Consistently, the co-culture of MM cells with OCs significantly increased CD26/DPPIV secretion compared with the mono-culture of MM cells (Fig. 2e). The co-culture additionally increased CD26/DPPIV production 1.8- to 2.5-fold compared with OC culture (Fig. 2e). These results demonstrate that CD26 is overexpressed in both MM cells and OCs in their co-culture system. We also defined target genes in MM cell lines by real-time quantitative RT-PCR analysis. CD26 mRNA expression in MM cell lines co-cultured with OCs revealed 7- to 19.4-fold increase compared with those co-cultured alone (Fig. 2f).

Next, to test whether direct contact between MM cells and OCs is required for CD26 upregulation in MM cells, the co-culture system with or without direct contact was used. Immunohistochemistry showed that CD26 expression in MM cell lines (U266, KMS18, KMS26, KMS27, KMS28, and KMS34) co-cultured with OCs without direct contact was also increased as well as those in the co-culture with direct contact (Figure S2). Further, to elucidate the factors involved in the regulation of CD26 expression in MM cells, MM cell lines (KMS18, KMS26, and KMS28) were cultured under stimulation with anti-apoptotic cytokines, including IL-6, SDF-1, TNFα, APRIL, and BAFF, produced by the OCs or BMSCs in MM. and these cytokines enhanced CD26 expression in the MM cells (Figure S3). These results support that soluble factors such as IL-6 and TNF family cytokines may be certain factors responsible for CD26 upregulation in MM cells.

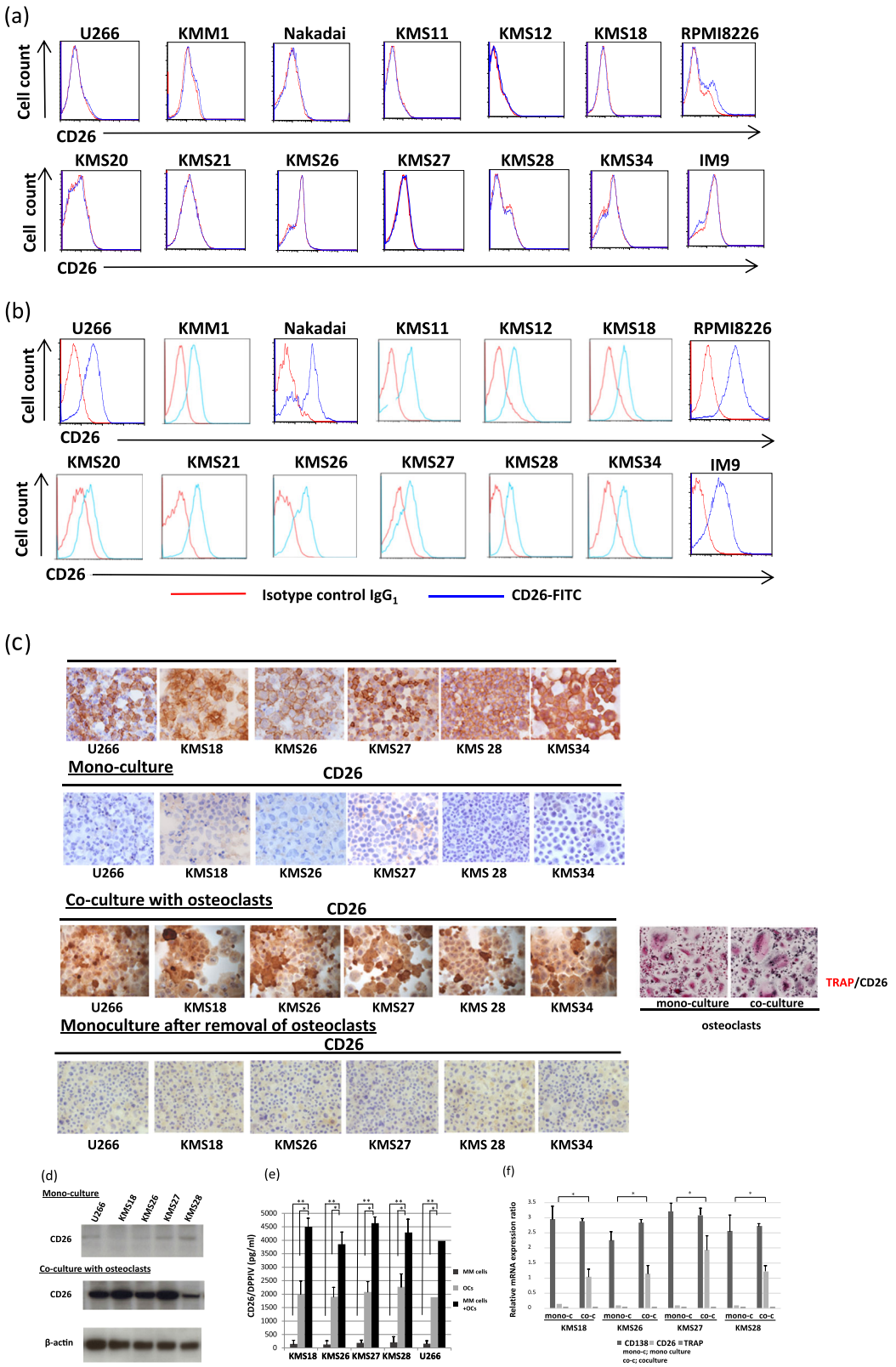

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Fig. 2 CD26 expression in MM cell lines. a, **b** Flow cytometry with anti-CD26 (rat clone)-FITC (blue histogram) or isotype control IgG_1 (red histogram) was performed in 14 MM cell lines; U266, KMM1, Nakadai, KMS11, KMS12, KMS18, RPMI8226, KMS20, KMS21, KMS26, KMS27, KMS28, KMS34, and IM9. Open histograms to the left of each panel represent iso IgG_1. CD26 expression on **a** MM cell lines cultured alone (mono-culture) and **b** MM cell lines co-cultured with human OCs for 72 h is shown. **c** MM cell lines (U266, KMS18, KMS26, KMS27, and KMS28) cultured alone or recovered from co-culture with OCs were stained with CD138 and CD26 by immunohistochemistry (Original magnification, ×100). All tested MM cell lines expressed CD138 regardless of mono-culture or co-culture (data not shown) with OCs. In contrast, MM cell lines cultured alone lacked CD26 expression. However, after co-culture with OCs, CD26 expression was upregulated in MM cell lines. Moreover, when subsequently cultured alone for 48 h after removal of co-culture with OCs, CD26 expression in MM cell lines was downregulated again. OCs, both cultured alone and recovered from co-culture, expressed CD26 (TRAP; red-stained, CD26; gray-stained) (original magnification, ×100). **d** CD26 expression in U266, KMS18, KMS26, KMS27, and KMS28 cultured alone (mono-culture) or co-cultured with OCs was examined by immunoblot analysis. Cell lysates were harvested from each cell lines. β-actin was blotted as a loading control. **e** The level of CD26/DPPIV derived from supernatants in mono-culture of OCs or MM cell lines and those in the co-culture of OCs with MM cell lines was measured by ELISA. The CD26/DPPIV level in supernatants from the mono-culture of OCs or the co-culture of MM cell lines with OCs was significantly elevated compared with those from the mono-culture of MM cell lines. The data represent the mean ± SE of triplicate wells from the representative of three independent experiments. The error bars represent the range (*$p < 0.05$. **$p < 0.01$). **f** The expression of target genes (CD138, CD26, and TRAP) in MM cell lines (KMS18, KMS26, KMS27, and KMS28) co-cultured with OCs or cultured alone was assayed by real-time quantitative RT-PCR using specific primers. CD26 mRNA expression in MM cells co-cultured with OCs revealed 7- to 19.4-fold increase compared with those co-cultured alone. The results are shown as ratio of indicated gene mRNA/β-actin mRNA. Data represent the the mean ± SE. $n = 3$. *$p < 0.05$

Humanized anti-CD26 monoclonal antibody (huCD26mAb) inhibited the survival of CD26+ MM cells at higher concentrations

First, to clarify the role of CD26 in MM cell survival, we examined the impact of inhibition by huCD26Ab on MM cell growth. OCs derived from human BM-MNCs were co-cultured with the MM cells in 24-well plates for 72 h. Next, the cells were incubated in the presence of the indicated concentrations of huCD26mAb (0.1, 1.0, 10, 50, or 100 μg/ml) or isotype control IgG_1 for 48 h, and viable MM cells were evaluated. huCD26mAb had little or no direct effect on CD26− MM cell lines cultured alone, but it directly and dose-dependently inhibited the growth of CD26+ MM cells co-cultured with OCs primarily at higher concentrations >10 μg/ml (Fig. 3a).

huCD26mAb revealed significant anti-MM efficacy by ADCC against CD26+ MM cells but not CD26− MM cells

Next, we conducted a calcein-AM release assay to examine the ability of huCD26mAb to lyse MM cells via ADCC. huCD26mAb-triggered ADCC against tested CD26+ MM cell lines (U266, KMS18, KMS26, and KMS28) co-cultured with OCs in the presence of NK effector cells from three different donors in an effector–target (E/T) ratio (0.2, 1.0, 5.0, 25, or 50)-dependent manner (Fig. 3b). At an E/T ratio of 20:1, huCD26mAb induced the lysis of CD26+ MM cell lines co-cultured with OCs, with lytic activity initiating at a huCD26mAb concentration of 0.0001 μg/ml and maximum lysis at 10 μg/ml by ADCC (Fig. 3c, S3). In contrast, huCD26mAb did not exhibit dose-dependent ADCC against CD26- MM cell lines cultured alone (Figure S4). These results demonstrated that the immune mechanisms of action of huCD26mAb though ADCC activity against CD26+ MM cells.

Treatment with huCD26mAb in conjunction with conventional or novel agents synergistically augmented ADCC against CD26+ MM cells, compared with huCD26mAb alone

We further explored whether pretreatment with conventional therapy (dexamethasone: Daexa) or novel agents (bortezomib: BTZ, lenalidomide: Lena) facilitates huCD26mAb-induced ADCC against CD26+ MM cells. CD26+ MM cell lines (KMS18, KMS26, and KMS28) were co-cultured with OCs and pretreated with Dexa (25 nM), BTZ (3 nM), and Lena (0.5 μM) for 24 h. Pretreatment with Dexa or BTZ significantly augmented subsequent MM cell lysis by NK effector cell-dependent ADCC by huCD26mAb (10 μg/ml) at the E/T ratio of 20 (Fig. 4a). Additionally, CD26+ MM.1 R cell lysis, following pretreatment with Dexa, BTZ, and Lena (0.05, 0.5 μM) was enhanced by huCD26mAb-induced ADCC (Fig. 4b). Lenalidomide is an immunomodulatory drug that was previously shown to increase NK cell activity. Pretreatment of NK effector cells with Lena further augmented subsequent huCD26mAb-induced lysis of CD26+ MM.1R cells in a dose-dependent manner (Fig. 4c). These results suggest that huCD26mAb induces significant ADCC against MM cells, regardless of sensitivity or resistance to conventional or novel agents, and that the addition of novel agents to huCD26mAb increased sensitivity to huCD26mAb-induced ADCC against CD26+ MM cells.

huCD26mAb did not exhibit dose-dependent complement-dependent cytotoxicity (CDC) lysis against CD26+ MM cells

We evaluated the effect of CDC by huCD26mAb against CD26+ MM cells. In the presence of 50% human serum as a source of complement, human anti-HLA-DR induced

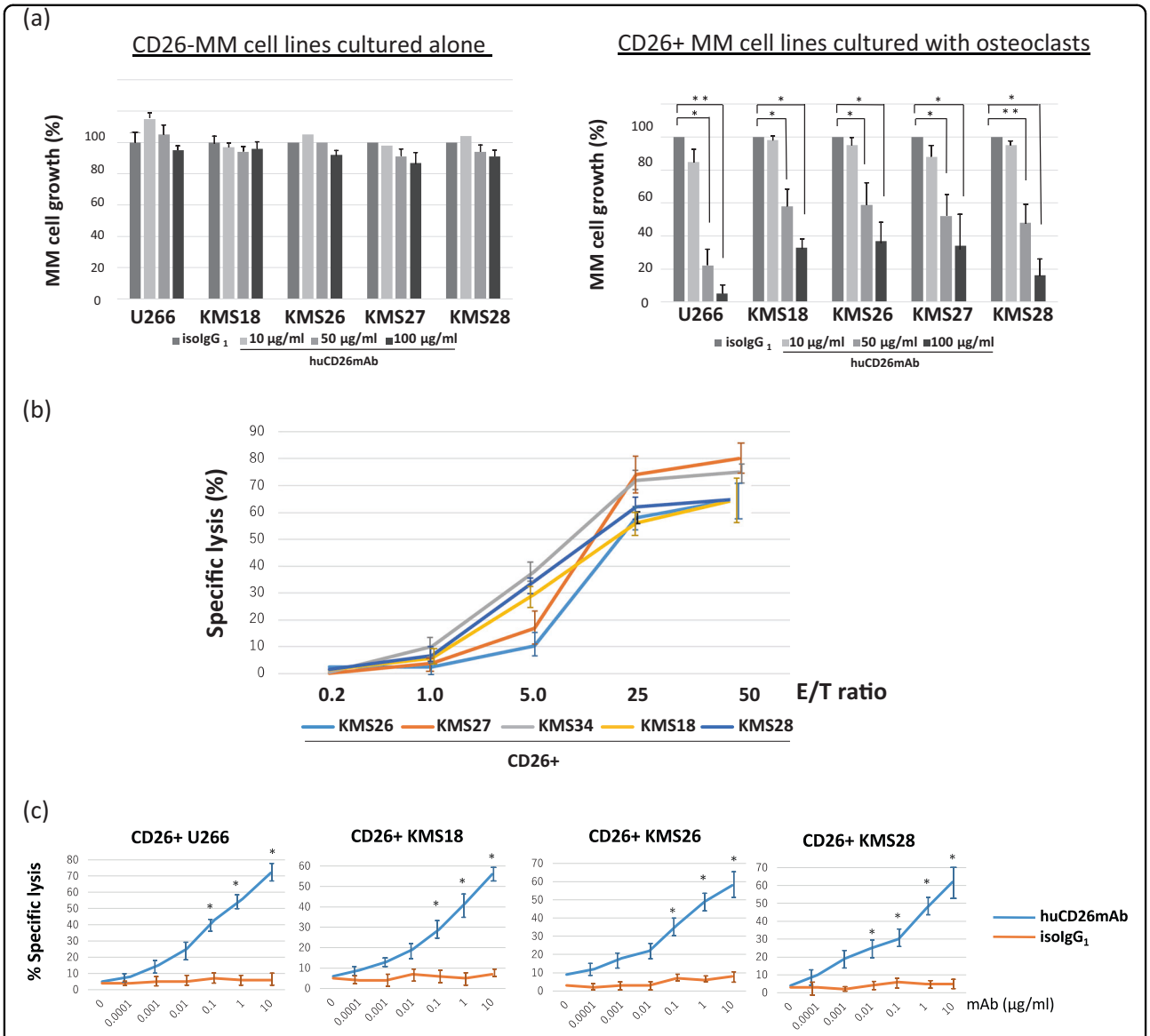

Fig. 3 huCD26mAb exhibited ADCC against CD26+ MM cell lines. a Cell viability was determined usingct effects on the growth of CD26− MM cell lines cultured alone (left panel), but it dose-dependently inhibited the proliferation of CD26+ MM cells in co-culture with OCs primarily at higher concentrations >10 μg/ml (right panel). The data represent the mean ± SE of three independent experiments (*$p < 0.05$, **$p < 0.01$). **b** ADCC assay was performed by co-culture of calcein-AM-labeled MM target cell lines with human NK effector cells in various effector–target (E/T) ratios, at a huCD26mAb concentration of 10 μg/ml. Specific lysis percentage (%) was calculated, and the data represent three experiments conducted with three different human NK effector cell donors. huCD26mAb-triggered CD26-specific lysis of CD26+ MM cell lines co-cultured with OCs in an effector–target (E/T) ratio-dependent manner by ADCC. **c** huCD26mAb induced lysis of CD26+ MM cell lines co-cultured with OCs with human NK effector cells at an E/T ratio of 20:1 in the presence of indicated concentrations of huCD26mAb (0.0001–10 μg/ml) or iso IgG_1. The data represent the mean ± SE of triplicate wells (*$p < 0.05$)

significant cell lysis, while huCD26mAb demonstrated a low or no potential to confer CDC against CD26+ MM cell lines (Fig. 5). Antibody-independent cytotoxicity (in the presence of serum without antibodies) was not observed. huCD26mAb treatment did not alter complement-regulatory protein expression, including CD55 or CD59, on these tested MM cell lines (data not shown).

huCD26mAb blocked CD26+ MM cell adhesion to BMSCs

To determine whether huCD26mAb exerts effects on CD26-mediated MM cell adhesion to BMSCs, we examined the impact of huCD26mAb on MM cell adhesion to BMSCs. Initially, MM cell lines (KMS18, 26, and 28) were co-cultured with BMSCs for 48 h. Subsequently, immunofluorescence staining showed increased CD26 expression in these cell lines (Fig. 6a). Next, calcein-AM labeled

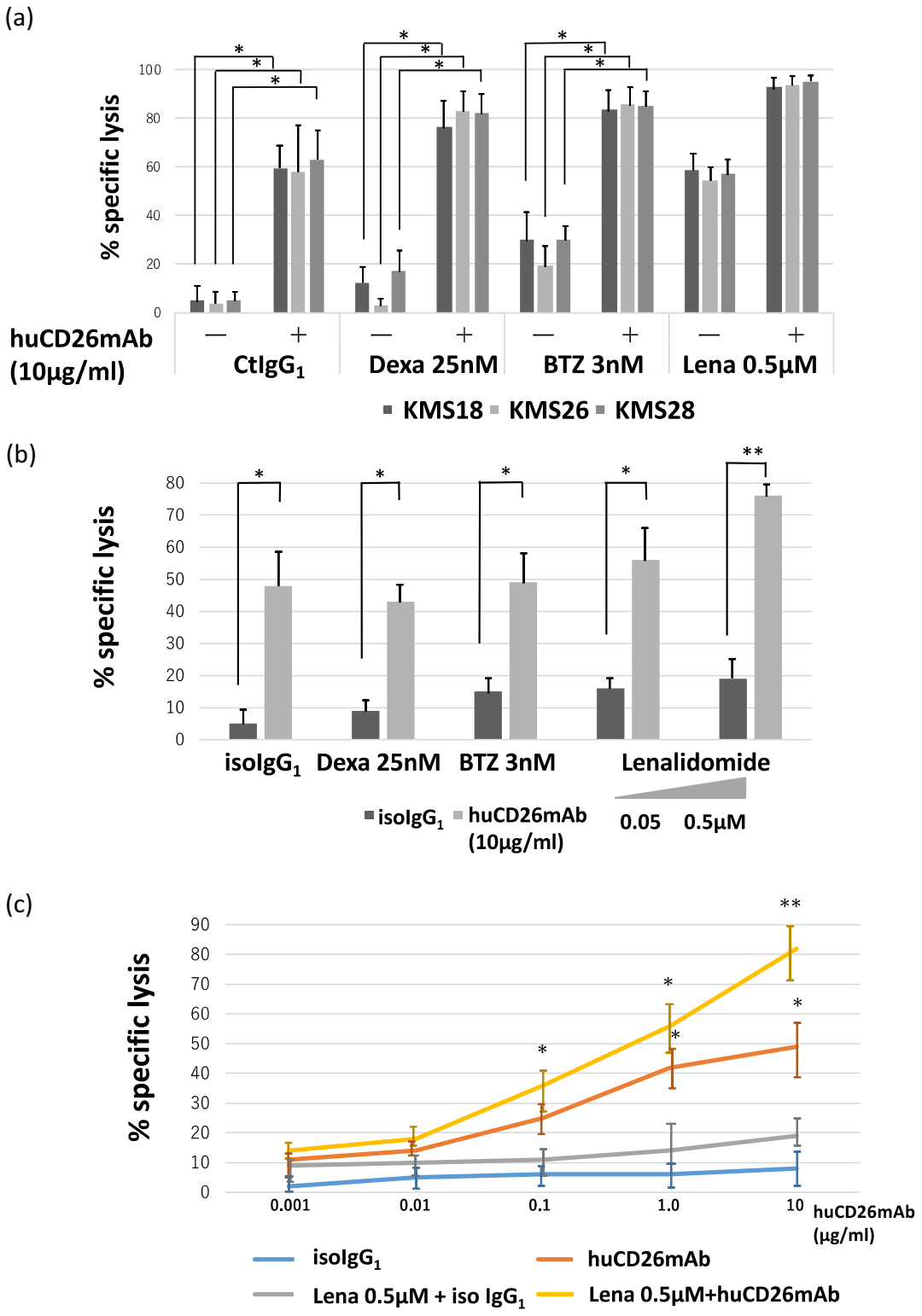

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Fig. 4 Combination of huCD26mAb plus novel agents induced significant lysis of CD26+ MM cell lines by ADCC. CD26+ MM cell lines; KMS18, KMS26, and KMS28, co-cultured with OCs were pretreated with dexamethasone (Dexa, 25 nM), bortezomib (BTZ, 4 nM), and lenalidomide (Lena, 0.5 μM) for 24 h. Then, calcein-AM-labeled CD26+ target MM cell lines were co-cultured with NK effector cells at the E/T ratio of 20 in the presence of huCD26mAb (10 μg/ml) or iso IgG_1. The data represent the mean ± SE of triplicate wells (*$p < 0.05$). **b** CD26+ MM.1R co-cultured with OCs was pretreated with Dexa (25 nM), BTZ (3 nM), or Lena (0.5 μM). Next, huCD26mAb-triggered ADCC lysis against CD26+ MM.1R in the presence of NK effector cells was assayed by calcein-AM release assay. The data represent the mean ± SE of triplicate wells (*$p < 0.05$, **$p < 0.01$). **c** NK effector cells were pretreated with Lena (0.5 μM) for 24 h followed by huCD26mAb-induced ADCC against CD26+ MM.1R cells cultured with OCs. The data represent the mean ± SE of triplicate wells (*$p < 0.05$, **$p < 0.01$)

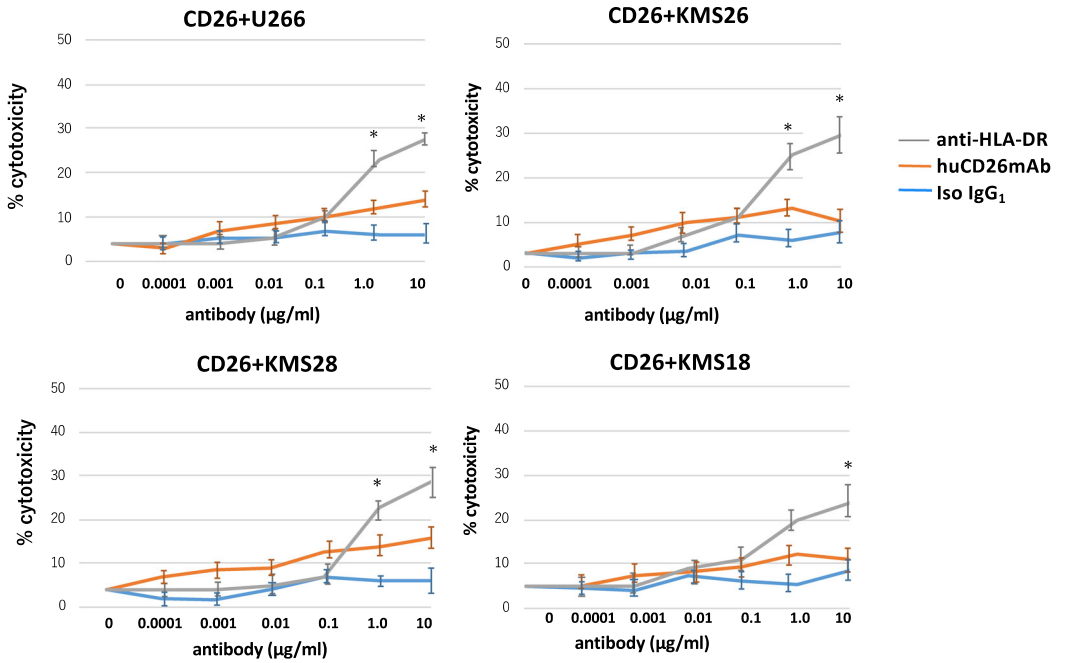

Fig. 5 huCD26mAb did not induce CD26+ MM cell lysis by CDC. CDC assay against CD26+ KMS18, KMS26, KMS28, and U266 co-cultured with OCs by huCD26mAb was performed using 50% human serum as a source of complement. Human anti-HLA-DR was used as a positive control and an iso IgG_1 as a negative control. The % cytotoxicity was measured by a calcein-AM release assay after a 1-h incubation of target cells with the indicated concentrations of huCD26mAb (0.0001–10 μg/ml) or iso IgG_1 in the presence of 50% human serum. In the presence of human serum, human anti-HLA-DR induced significant cell lysis. In contrast, huCD26mAb revealed barely or no remarkable CDC lysis compared with iso IgG_1 against CD26+ MM cells

MM cell lines (KMS18, KMS26, and KMS28) were added to BMSC-coated plates in the presence of the indicated concentrations of huCD26mAb (0.1, 1.0, 10 μg/ml) or iso control IgG_1 and incubated for 4 h. After removing and detaching non-adherent cells, adherent cells were quantified using a fluorescence microplate reader. huCD26-mAb dose-dependently inhibited the adhesion of MM cell lines to BMSCs: however, the iso control IgG_1 did not (Fig. 6b). We further tested the impact of huCD26mAb-induced ADCC against MM cells adherent to BMSCs. huCD26mAb (10 μg/ml) additionally exerted ADCC against MM cells in the presence of BMSCs as well as in the presence of OCs. These results indicate that huCD26mAb inhibits MM cell growth adherent to BMSCs, partly by blocking cell-to-cell contact between the BMSCs and MM cells (Fig. 6c). In addition, the cell surface expression of CD49d (α4-integrin, a subunit of VLA4) was significantly reduced in MM cells recovered from co-culture with BMSCs following huCD26mAb treatment (10 μg/ml) in the presence of NK effector cells compared with untreated MM cells (Fig. 6d).

huCD26mAb inhibits both CD26+ MM cell growth and MM-related osteolytic bone disease in vivo

We further examined whether huCD26mAb can suppress MM tumor burden and its related bone disease in vivo. We used two xenograft models: a subcutaneous tumor model of MM and an intra-bone tumor model of MM. Initially, CD26+ KMS18 cells prepared by co-culture with OCs in vitro were subcutaneously inoculated into the 6-week-old female NOD/SCID mice (Fig. 7a). Within 10 days of implantation, tumors became visible at

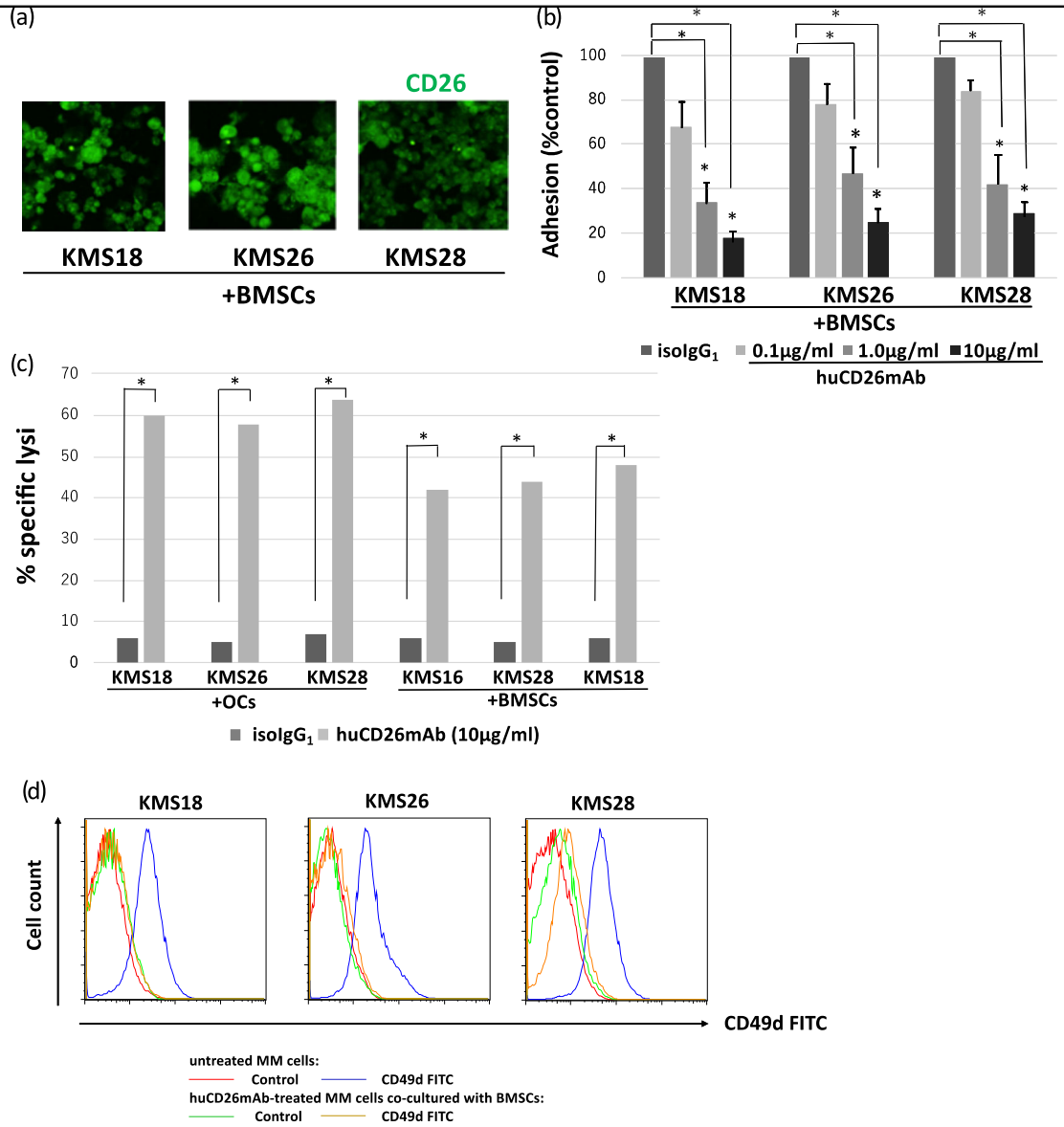

Fig. 6 huCD26mAb blocked CD26+ MM cell adhesion to BMSCs. a After MM cell lines (KMS18, KMS26, and KMS28) were co-cultured with BMSCs for 48 h, the MM cells were collected and incubated. Next, cytospin slides using these MM cell lines were fixed with 4% PFA and were incubated with anti-human CD26 polyclonal antibody and Hoechst 33342 (5 μg/ml), washed and fixed. Images were processed using Nanozoomer-XR. MM cells co-cultured with BMSCs were positive for CD26. **b** The % adhesion of calcein-AM-labeled KMS18, KMS26, and KMS28 cells added to BMSCs was measured in the presence of huCD26mAb (0.1, 1.0, 10 μg/ml) or iso IgG_1. huCD26mAb dose-dependently inhibited the adhesion of MM cell lines to BMSCs, while iso IgG_1 did not. The data represent the mean ± SE of triplicate wells from three independent experiments (*$p < 0.05$). **c** huCD26mAb-triggered ADCC against MM cell lines (KMS18, KMS26, and KMS28) in the presence of BMSCs versus OCs was examined by a calcein-AM release assay. The data represent the mean ± SE of three independent experiments (*$p < 0.05$). **d** Cell surface CD49d expression in MM cells following huCD26mAb treatment was tested in the presence of NK effector cells. After a 2-h incubation, cells were harvested to determine the expression level of CD49d on MM cells by flow cytometry. CD49d expression was significantly reduced in huCD26mAb (10 μg/ml)-treated MM cells (KMS18, KMS26, and KMS28), recovered from co-culture with BMSCs compared with untreated MM cells

the injection site. The subcutaneous tumors decreased in both weight and size in the huCD26mAb-treated mice compared with those of the control mice (mean weight: 1.1 ± 0.5 versus 5.7 ± 0.6 g, $p < 0.05$, on day 29) (Fig. 7b, c). Next, human bone grafts were subcutaneously implanted in the NOD/SCID mice, and KMS18 cells cultured alone were directly injected into the human bone grafts (NOD/SCID-hu mice) (Fig. 7d). CD26 expression in the KMS18 cells was increased through the interaction between the KMS18 cells and the BM microenvironment, including BM accessory cells such as OCs osteogenic cells, endothelial cells, adipocytes, fibroblasts, and BMSCs.

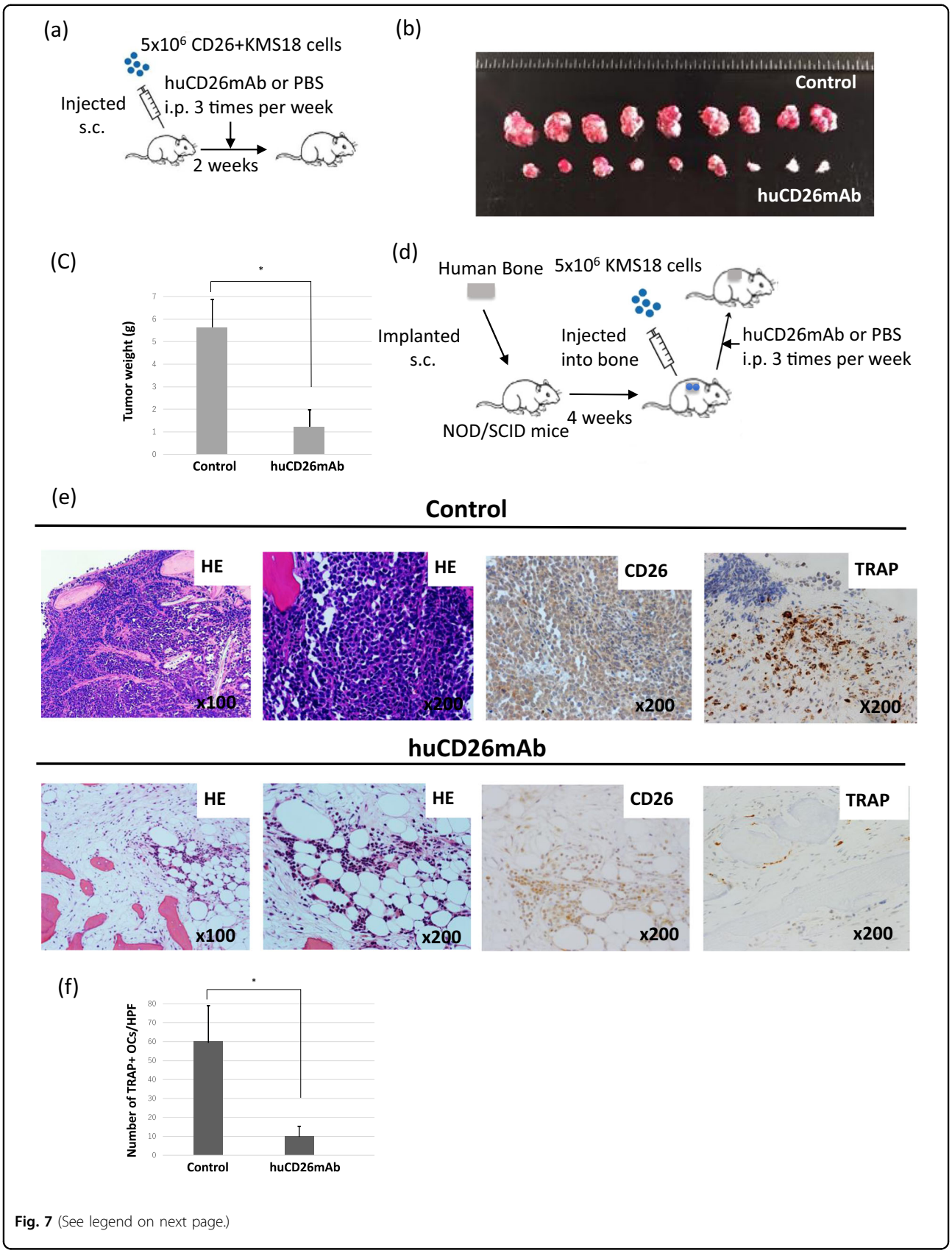

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Fig. 7 Anti-MM activity of huCD26mAb *in vivo*. a The NOD/SCID model of MM. The NOD/SCID mice were subcutaneously inoculated with KMS18 cells that were co-cultured with OCs (5×10^6 cells per mouse). After 2 weeks, the tumors were established, and the mice were randomized into two groups. The mice ($n = 5 \times 2$) were treated either PBS or huCD26mAb (10 mg/kg/dose) i.p. three times a week for 2 weeks. **b** Direct in vivo anti-tumor activity of huCD26mAb was compared with the control. Tumor specimens in PBS-treated versus huCD26mAb (10 mg/kg/dose)-treated MM-bearing NOD/SCID mice on study day 15 were shown. **c** The average tumor weights at necropsy in PBS-treated (control) versus huCD26mAb-treated mice were measured on study day 29. huCD26mAb significantly decreased the tumor weights compared with the control. The data are shown as the mean ± SD of three independent experiments. (*$p < 0.05$). **d** The NOD/SCID-hu model of MM. KMS18 cells cultured alone (5×10^6 cells per mouse) were directly injected into the human bone and subcutaneously implanted in the NOD/SCID mice. 4 weeks after tumor injection, when BM engraftment was confirmed, the mice were randomized into two groups. The mice ($n = 5 \times 2$) were then treated with either PBS or huCD26mAb (10 mg/kg/dose) i.p. three times per week for 4 weeks. **e** huCD26mAb inhibits both CD26+ MM tumor burden and OC formation in vivo. Histology and immunohistochemical findings from the collected human bones were shown (from the left panel: H&E, ×100, ×200; CD26 stain, ×200; TRAP stain, ×200). Continuous treatment with huCD26mAb significantly inhibited CD26+ MM cell growth. In addition, a number of TRAP+ OCs were observed in the control mice, whereas huCD26mAb markedly decreased the number of TRAP+ OCs in human bones of the huCD26mAb-treated mice. huCD26mAb inhibits both MM tumor burden and OC formation in vivo. **f** The number of TRAP+ OCs in the human bones was quantified under three random fields at ×100 magnification. The number of OCs was significantly lower in huCD26mAb-treated mice than in the control mice (*$p < 0.05$)

Histological examination by hematoxylin eosin staining of the bones of the control mice revealed massive infiltration of MM cells into the bone cavity compared with those of huCD26mAb-treated mice (Fig. 7e). In addition, immunohistochemical analysis with CD26 confirmed decreased numbers of CD26+ MM cells in the human bone grafts retrieved from huCD26mAb-treated versus control mice (Fig. 7e). These results suggest that huCD26mAb has the potential to reduce CD26+ MM tumor burden in both xenograft models in vivo. Subsequently, we assessed the effect of huCD26mAb on MM-bone disease in the NOD/SCID-hu model. huCD26mAb suppressed TRAP+ OC formation in human bones and the number of OCs was markedly decreased in the huCD26mAb-treated mice compared with the control mice (Fig. 7f). No adverse effects of huCD26mAb were observed in the BM of mice (data not shown). No mice died of progressive MM during the observation period. These results strongly suggested that huCD26mAb suppresses tumor progression and osteolytic bone disease in the BM milieu of MM.

huCD26mAb, alone or in conjunction with lenalidomide, reduced the side population (SP) ratio in CD26+ MM cells by ADCC

SP cells have been identified as a drug-resistant fraction by their ability to efflux a Hoechst 33342 dye, a substrate for the ATP-binding cassette (ABC) transporter, ABCG2[38]. We analyzed the expression of the SP and main population (MP) fractions in MM cell lines. RPMI8226 and KMS11 exhibited SP fractions, both of which in co-culture with OCs equally expressed CD26 at a high level as MP fractions (Fig. 8a). To determine whether monotherapy with huCD26mAb, Lena, or huCD26mAb in conjunction with Lena affect SP fractions in CD26+ MM cells, we further investigated the impact of these reagents on the ratio of SP fractions in these cells by ADCC. RPMI8226 and KMS11, co-cultured with OCs were incubated with NK effector cells at the E/T ratio of 10 in the presence of Lena (5 μM), huCD26mAb (10 μg/ml), or both in combination for 24 h. Subsequently, SP analysis was performed by flow cytometry. Indeed, although Lena alone did not reduce the ratio of the SP fraction in CD26+ RPMI8226 or KMS11, huCD26mAb substantially reduced its ratio in both of MM cell lines. Moreover, the SP ratio was further decreased by combination therapy with huCD26mAb plus Lena (Fig. 8b, c). These results suggested that the intense CD26 expression on the SP fractions of CD26+ MM cells could be a therapeutic target by huCD26mAb.

Discussion

MM has a unique property to develop and expand within the BM and causes osteolytic bone destruction. OCs also support MM cell growth and protect MM cells from drug-induced apoptosis by creating a MM-specific BM microenvironment[1,32,33,39]. Our recent work has revealed that CD26 is intensely expressed on activated OCs in MM, and huCD26mAb inhibits human OC differentiation[29]. Furthermore, immunostaining on bone marrow biopsy specimens showed that CD26 is expressed on plasma cells around OCs in MM patient. Accordingly, the aims of this study were to examine in detail the expression profile of CD26 in MM cells through the interaction with the BM microenvironment and elucidate the anti-MM efficacy of huCD26mAb as a therapeutic antibody for MM. The current study is the first report to show CD26 as a potential novel antibody-based therapeutic target in MM.

Initially, the interaction of MM cells with OCs or BMSCs identified that CD26 is highly expressed and prevalent in both co-cultured MM cells and OCs as demonstrated by immunohistochemistry. Additionally, a high level of CD26/DPPIV expression was detected in the supernatants obtained from both co-culture of MM cells with OCs and OC culture compared with those obtained from the MM cell mono-culture. These results suggest

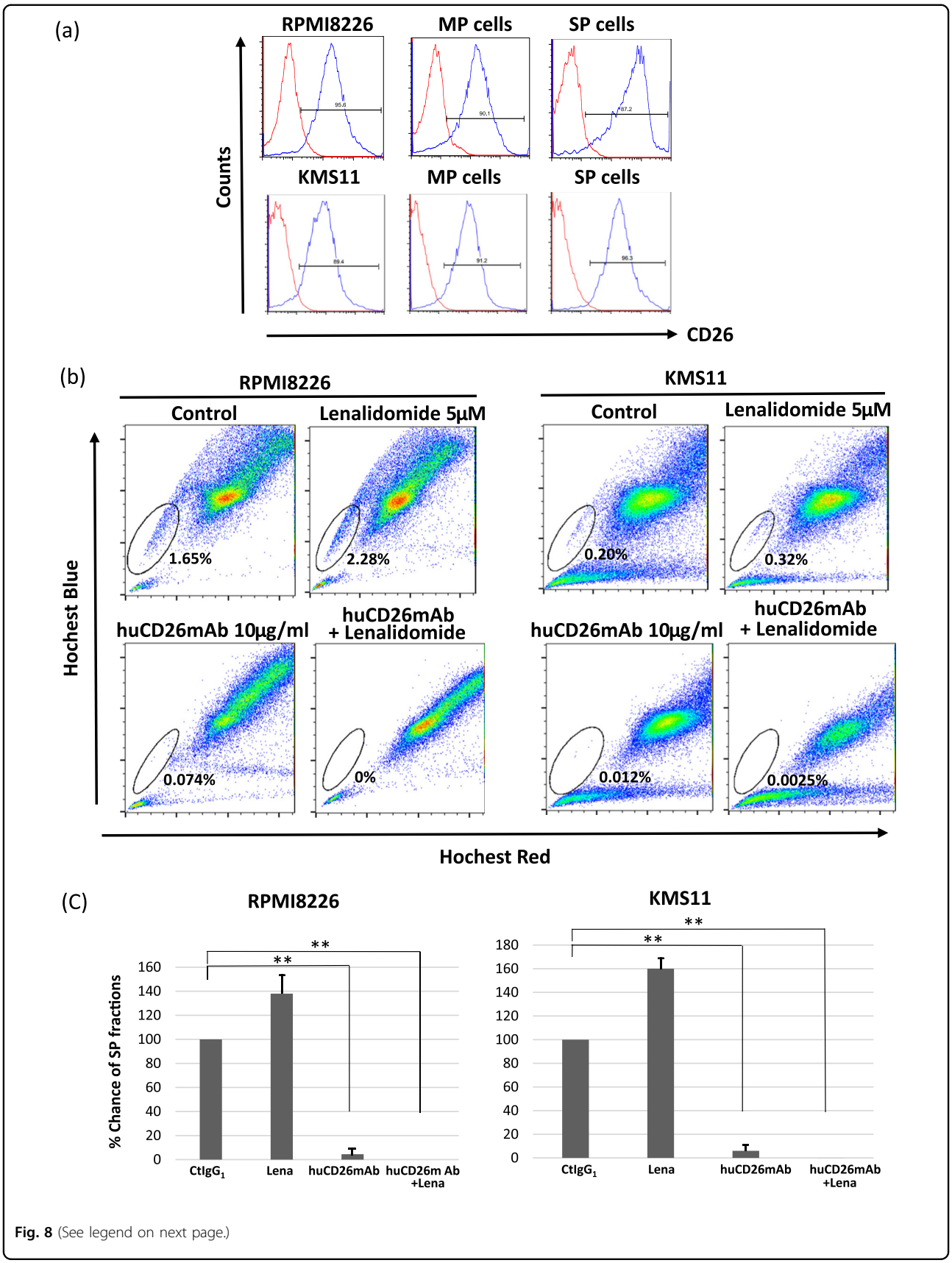

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Fig. 8 huCD26mAb, alone or in combination with Lena, reduced the SP ratio by ADCC. a SP fractions were determined by Hoechst 33342 dye and gated as indicated in both RPMI8226 and KMS11 co-cultured with OCs. Then, SP and MP fractions were isolated from RPMI8226 and KMS11 by cell sorter and the expression of CD26 in both SP and MP fraction was analyzed by flow cytometry. SP fractions equally expressed CD26 at a high level as MP fractions in both cell lines. **b** RPMI8226 and KMS11 co-cultured with OCs were mixed with NK effector cells from health donor at the E/T ratio of 10 in the presence of iso IgG_1, Lena (5 μM) or huCD26mAb (10 μg/ml), alone or both in combination for 24 h. Thereafter, the ratio of SP fractions within the whole living cells was examined by flow cytometry. huCD26mAb reduced SP ratio in both MM cell lines through ADCC. In addition, it markedly decreased its ratio with Lena in combination. **c** The % change of SP fractions after the indicated treatments (monotherapy of Lena or huCD26mAb, or Lena plus huCD26mAb in combination), mixed with NK effector cells are revealed. The data represent the mean ± SD of three independent experiments (**$p < 0.01$)

the significance of CD26 in MM cells through the interaction with BM microenvironment[40]. Importantly, ex vivo experiments demonstrated that ADCC is the leading mode of action of huCD26mAb in MM. huCD26mAb exerted potent anti-MM efficacy via immune effector mechanism by ADCC in addition to direct on-tumor effects, which resulted in potent cytotoxicity against CD26 + MM cells. Moreover, the ADCC effect by huCD26mAb was synergistically facilitated in conjunction with first-generation novel agents, such as Lena and BTZ. NK effector cells involved in ADCC were known to be clearly inhibited by the exposure to steroids, while pretreatment of the target cells with Dexa increased lysis by ADCC, and the simultaneous treatment with Dexa plus rituximab did not impair ADCC in malignant lymphoma[41]. These previous reports support our results showing cytotoxic synergy with Dexa plus huCD26mAb in MM.

Moreover, a role for anti-MM efficacy by huCD26mAb was supported by subsequent in vivo experiments. The NOD/SCID-hu model is an in vivo system, which makes it possible to evaluate both tumor burden and bone disease via providing a favorable host environment for reproducible growth of MM cells and mimicking the clinical manifestations of MM. Thus, this model is widely used to analyze MM biology and develop novel therapeutic strategies for MM[33–37,39,42]. This model is quite unique in the point that both human hematopoietic cells and the human hematopoietic microenvironment are engrafted in the mice[43,44]. Bone marrow accessory cells including OCs and stromal cells in the human bone grafts can support sustained growth of MM cells[35]. Moreover, human stromal cells in the bone grafts can stimulate the proliferation and differentiation of human stem cells through hematopoietic factors or physical interactions and multilineage human hematopoiesis is maintained within implanted human bone grafts of the mice for as long as 20 weeks after implantation[44]. Thus, human immune cells including T lymphocytes or NK cells are considered to be able to confer the inhibitory effects against CD26+ MM cells by huCD26mAb as well as huCD26mAb exerts direct inhibitory effects against CD26+ MM cells in the human bone marrow of NOD/SCID-hu mice. In addition, our results confirmed that huCD26mAb induced a significant decrease in the number of TRAP+ OCs compared with control mice in vivo. These results have clinical relevance, indicating that huCD26mAb appears to be an ideal therapeutic antibody to target both CD26+ MM cells and OCs for the treatment of MM.

Although immune-based approaches have not succeeded in MM until recently, these strategies have finally attained an exciting breakthrough and represent a promising area for novel therapeutic options. Target antigens for mAb therapy in MM have been either cell surface proteins, cytokines, or chemokines, expressed or secreted by MM cells, including growth factors, signaling molecules, and adhesion molecules, which are involved in cell growth, anti-apoptosis, angiogenesis, and cell-to-cell contact between MM cells and BM accessory cells[2,9,11,12]. In addition to elotuzumab targeting CS1 and daratumumab directed against CD38, other monoclonal antibody targets include CD138, CD56, CD40, CD74, and intercellular adhesion molecule-1 (ICAM-1) among cell surface targets as well as IL-6, vascular endothelial growth factor (VEGF), BAFF, and CXC chemokine receptor 4 (CXCR4) for cytokine/growth factor-targeted molecules, which have reached clinical trials[9,11,12].

CD26 regulates a variety of cytokines and chemokines through the cleavage of N-terminal dipeptides from polypeptides, with proline or alanine in the penultimate position. CD26 might be involved in regulating the activity of biopeptides to have a key role in tumor cell survival and proliferation[28]. Likewise, in our study, CD26 activity was important for both OC formation and OC-induced MM cell growth. Therefore, the inhibition of CD26 could be beneficial for the treatment of MM.

CD26 expression in tumor cells reveals varying results. It acts as a tumor activator or tumor suppressor and may either promote or suppress growth depending on the type of malignancy. Indeed, the presence of CD26 is associated with clinical aggressiveness in several tumors, while the

absence of CD26 results in distant metastasis in others. In hematological neoplasms, CD26 expression confers proliferative advantages or invasive properties in T-cell lymphoblastic lymphoma (LBL)/lymphoblastic leukemia, T-anaplastic large cell lymphoma (ALCL), and T-large granular lymphocyte lymphoproliferative disorder (T-LGL LPD)[45–47]. In addition, CD26 expression, together with CD38 and CD49d expression, identified B-CLL patients with an unfavorable clinical outcome[48]. In solid tumors, CD26 has a reliable biomarker of gastro-intestinal stromal tumor (GIST) risk grading[49]. Our previous studies showed that CD26 expression in mesothelioma cells as well as renal cell carcinoma (RCC) cells was associated with enhanced proliferative activity[50–53]. In addition, our previous reports showed that anti-CD26 mouse mAb (IgG_1), IF7 or 14D10, inhibited tumor cell growth of CD26 + T-cell lymphoma or RCC[50,54]. huCD26mAb, constructed from a 14D10 coding sequence, has been shown to inhibit malignant mesothelioma cell growth[51–56]. On the other hand, the inhibition of CD26 triggers prostate cancer metastasis. The degradation of SDF-1/CXCL12, known to regulate prostate cancer cell metastasis by CD26, is involved in the metastatic cascade of prostate cancer[57].

Moreover, CD26 has been indicated to have an important role in cell adhesion to the extracellular matrix (ECM) in selected conditions, which leads to the migration and metastasis of various types of tumors[28,50,54]. As a result of CD26 depletion through the transfection of interfering RNA, T-ALCL cells lost the ability to adhere to fibronectin and collagen I through the dephosphorylation of both integrin β1 and p38 mitogen-activated protein kinase (MAPK), which suppresses tumor development in in vivo xenograft models[58]. In addition to cytotoxic effects on MM cells, we validated that huCD26mAb induced inhibitory effects on CD26+ MM cell adhesion to BMSCs, which partly conferred to inhibit MM cell growth. Although huCD26mAb does not reduce expression of all adhesion molecules, it reduced the expression of membrane protein, CD49d in MM cells in the presence of effector cells.

The normal tissue profile of CD26 appears to be inevitably a major concern for tissue toxicities for an antibody therapy. A phase 1 clinical trial of huCD26mAb has been performed in patients with 33 cases of advanced CD26-expressing tumors, such as RCC, mesothelioma, and urothelial carcinoma[59]. In this trial, huCD26mAb was well tolerated with the major adverse events being infusion reactions. Owing to the expression of CD26 in normal hematopoietic cells, a decrease in the levels of peripheral lymphocytes, including CD26+ lymphocytes, was noted within a few days after the administration of huCD26mAb: however, this decrease was resolved within a month.

SP cells are defined as an enriched source of cancer-initiating cells with cancer stem cell (CSC) properties, which have been identified in several tumors[38]. We identified that the treatment of CD26+ SP fractions in both CD26+ RPMI8226 and KMS11 cells with huCD26mAb revealed sensitivity to ADCC and that huCD26mAb in conjunction with Lena resulted in additive cytotoxicity by ADCC, indicating that huCD26mAb could eradicate drug-resistant CD26+ CSCs in MM. Interestingly, CD26 has been validated as a novel marker of CSCs in several malignancies[60,61]. First, CD34+/CD38 − leukemic stem cells (LSCs) derived from patients with the chronic phase (CP) of chronic myeloid leukemia (CML) expressed CD26 at high levels. Specifically, all CD34+/CD38−/CD26+ LSCs were positive for BCR/ABL, as determined by FISH, and exhibited CSC properties, such as long-term proliferation and repopulation activity in NSG mice, whereas CD26− LSCs exhibited none of these functions[60]. Second, CD26+ colorectal cancer (CRC) cells led to the development of distant metastasis, which were associated with increased invasiveness and resistance to chemotherapy, while CD26− CRC cells did not, suggesting the existence of CD26+ CSCs in CRC[61].

In summary, we identified CD26 expression not only in activated OCs but also in MM cells in the bone marrow tissue of MM patient or MM cell lines co-cultured with OCs in vitro. Novel huCD26mAb, directly targeting CD26, elicited significant anti-MM efficacy by impairing both CD26+ MM cells and OCs in vitro and in vivo. Moreover, huCD26mAb facilitated its activity in conjunction with the existing standards of care. Our results strongly suggest that CD26 could be an ideal therapeutic target of antibody-based therapy in MM.

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Author contributions

H.N. designed the research, performed the experiments, analyzed the data and wrote the manuscript; M.H. analyzed the data; C.M., and M.S. contributed to the

coordination of research; T.Y. analyzed the data, wrote the manuscript and directed the project.

Conflict of interest

C.M. and T.Y. are consultants for Y's Therapeutics and Kissei Pharmacceutical Co. Ltd. The remaining authors declare that they have no conflict of interest.

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