B. 研究方法

1）治療効果を予測するバイオマーカーの探索

これまでに、がん細胞の表面上に PD-1 リガンドが発現している場合には、抗 PD-1 阻害抗体が治療効果を示す可能性が高いことを示唆する結果が得られているが、リンパ球側のバイオマーカーについては、ほとんど解析されていない。そこで、リンパ球側のバイオマーカーを探索する目的で、まず T 細胞の活性化状態を可視化するシステムの構築を試みた。具体的には、NF-AT、NF κB、AP-1 等の転写因子が結合する配列を1個あるいは複数個重複させて EGFP、薬剤耐性遺伝子および CRE 組換え酵素の上流に配置したプラスミドベクターを作製した。また、CRE 組換え酵素により EGFP および薬剤耐性遺伝子を発現するプラスミドベクターを作製した。各発現プラスミドベクターを、PD-1 による抑制機能を細胞レベルで観察し得る DO11.10-T 細胞株に導入した後、特異的な抗原を用いて刺激し、蛍光タンパク質の発現頻度と強度および薬剤耐性能の獲得頻度を検討した。

2）抗 PD-1 阻害抗体と組み合わせることにより相乗効果を示す治療法の開発

PD-1 阻害は、がん免疫を増強する反面、自己免疫を惹起する可能性がある。PD-1 欠損マウスが、マウスの系統により異なる種類の自己免疫疾患を自然発症することから、PD-1 欠損による免疫応答の活性化には、PD-1 欠損以外の遺伝要因や環境要因が大きく影響を与えると考えられる。最近我々は、LAG-3（Lymphocyte activation gene-3）という別の免疫抑制受容体が PD-1 と協調的に自己免疫を制御していることを見出した。がん免疫における抗原は、多くの場合自己抗原そのものであるため、がん免疫においても PD-1 と LAG-3 が協調的に働くと考えられる。そこで、LAG-3 の機能を阻害し得る抗体を作製し、その機能を評価した。

（倫理面への配慮）

動物実験にあたっては「動物の愛護及び管理に関する法律」を遵守するとともに、徳島大学動物実験委員会において許可を得た後、徳島大学動物実験管理規則に則って行った。

遺伝子改変動物の使用等、組換え遺伝子実験にあたってはカルタヘナ議定書、遺伝子組換え生物等の使用等の規制による生物の多様性の確保に関する法律を遵守するとともに、徳島大学遺伝子組換え実験安全管理委員会において許可を得た後、徳島大学遺伝子組換え実験安全管理規則に則って行った。

C. 研究結果

1）治療効果を予測するバイオマーカーの探索

これまでに検討したものの中では、NF-AT 結合配列を 8 個導入したプラスミドベクターが、感度と特異度を総合して考えると最も良好な結果を示した。NF-AT 結合配列を 8 個導入したプラスミドベクターを導入した DO11.10-T 細胞株では、未刺激ではレポーター遺伝子を発現する細胞はほとんど存在せず、1 μM の抗原をパルスした IIA1.6-B 細胞株を用いて刺激した際に、約 30%の細胞がレポーター遺伝子を発現した。

2）抗 PD-1 阻害抗体と組み合わせることにより相乗効果を示す治療法の開発

これまでに 16 種類の抗マウス LAG-3 モノク

ローナル抗体を得ることに成功した。そのうち、6 種類が試験管レベルで良好な機能阻害活性を示した。そのうちの一つは、既に市販されている抗体(クローン名:C9B7W)と比較して、親和性が 10 倍以上高く、阻害活性もより高いことが確認された。

D. 考察

活性化 T 細胞を可視化するレポーターコンストラクトの作製に成功したが、より良い比較を実現するためには、標識率を向上させる必要がある。また、PD-1 によるレポーター誘導の抑制効率の検討や、マウス由来細胞を用いた評価等も順次行って行く予定である。

LAG-3 阻害抗体については、高い阻害活性を有するモノクローナル抗体を得ることができたため、各種がん免疫の実験系において、その効果を検討する予定である。

E. 結論

抗 PD-1 阻害抗体は、がんの治療に対して革新的な変化を与えると期待される。しかし、抗 PD-1 阻害抗体が全てのがん患者に対して効果を示す訳では無く、また全てのがん患者を完治させる訳では無いことから、抗 PD-1 阻害抗体が効果を示す患者の選別および PD-1 と併用する薬剤の開発が極めて重要であると考えられる。

F. 健康危険情報

なし

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 なし
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（難病・がん等の疾患分野の医療の実用化研究事業）
分担研究報告書

PD-1 欠損マウスを用いた、バイオマーカー探索の基礎的検討

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研究要旨

がんにおける、PD-1 阻害療法の可能性として起こり得る、自己免疫様症状と、その早期検出を可能にするバイオマーカーの開発、および環境危険因子を検討するため、PD-1 欠損マウスに既知の自己抗原を投与し、同時投与でT細胞反応に影響するような因子を検討した。その結果、抗原投与時に、結核死菌を同時投与すると、PD-1 欠損マウスは、野生型マウスよりはるかに強い自己抗原を認識する炎症細胞の分化を起こし、強い自己免疫様疾患を発症した。結核の不顕性感染や、それに類する微生物学的因子が疑われるがん患者においては、思わぬ炎症が起こる可能性があり、このような患者への、PD-1 阻害薬投与は、より慎重に行う必要があることが示唆された。

A. 研究目的

ヒト型 PD-1 抗体療法は、がんに対する有効な免疫増強治療法として国内外で精力的に開発が進められている。PD-1 阻害による免疫増強の副作用として、一部の患者には、自己免疫様症状が現れることが予測され、投与初期に自己免疫疾患の兆候をとらえるバイオマーカーを得ることが出来れば有用であると考えられる。また、自己免疫疾患の危険因子として、患者の先天性因子だけでなく、基礎疾患や不顕性感染といった環境因子が考えられる。当研究では、不顕性感染の代表である、結核菌の菌体抗原が危険性因子となる可能性を、PD-1 欠損マウスを用いて検討した。

B. 研究方法

PD-1 欠損マウスに、結核死菌単独、あるいは結核死菌と、モデル抗原（鶏アルブミン、あるいはマウス脳ミエリンタンパク由来）ペプチドを投与し、自己免疫様疾患の発症を 30 日間観察した。また、投与後、8 日後、30 日後のマウスよりT細胞を調整し、このT細胞を、免疫に用いたペプチド抗原で再刺激した。この培養上清を採集し、BD 社が提供する CBA アッセイを用いて、抗原特異的なサイトカイン産生を測定した。

（倫理面への配慮）

本研究は、京都大学医学研究科の定める動物実験実施要綱、および京都大学動物実験指針を遵守して行った。また、PD-1 欠損マウスは、組み換えDNA技術を用いて作出されており、事前に、京都大学組み換えDNA実験委員会の承認を得たうえで行った。

C. 研究結果

PD-1 欠損マウスにペプチドを免疫すると、野生型コントロールマウスに比べ、強いインターフェロンγの誘導が起こることがわかってい

る。免疫時に、結核死菌を同時投与したマウスでは、インターフェロンγに加えて、炎症性サイトカインである IL-17 の産生がみられ、PD-1 欠損マウスではこのさらなる増強が見られた。この炎症細胞産生は、外来抗原である鶏アルブミンおよび自己抗原であるマウス脳由来の抗原両方に対して見られたが、自己抗原の投与時には、マウスに強い自己免疫性脳脊髄炎が誘導された。PD-1 欠損マウスでは、結核死菌単独に対しても IL-17 産生細胞が産生された。

D. 考察

結核菌体は、トル様受容体を刺激して、抗原提示細胞による17型ヘルパーT細胞(Th-17)の誘導を起こす。PD-1 欠損下では、結核菌に対して誘導された Th-17 の増殖や免疫反応が増強し、自己抗原反応性のT細胞に対しても影響して、自己炎症性細胞への分化を促進する可能性が示唆された。

今後は、この実験系を用いて、自己免疫疾患発症初期の、血清生化学的、免疫学的パラメータなどを検討する予定である。

E. 結論

結核菌など、Th-17 を誘導する活性をもつ病原体、およびカビなどの環境因子は、潜在的に自己反応性 Th-17 の分化も起こす可能性を持っている。ここに PD-1 阻害を行うと、自己反応性 Th-17 の活性化を誘導し、思わぬ自己免疫病を発症する可能性がある。よって、患者における、不顕性感染などの微生物学的因子は、PD-1 抗体療法の際に慎重にモニタリングされる必要がある。

F. 健康危険情報

なし

G. 研究発表

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第 2 回日中韓免疫シンポジウム・大阪大学・2012 年 12 月 6 日

H. 知的財産権の出願・登録状況

(予定を含む)

1. 特許取得　なし
2. 実用新案登録　なし
3. その他　なし

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;なし

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Ⅳ. 研究成果の刊行物・別刷

UICC
global cancer control

IJC
International Journal of Cancer

The activated transforming growth factor-beta signaling pathway in peritoneal metastases is a potential therapeutic target in ovarian cancer

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Peritoneal dissemination including omental metastasis is the most frequent route of metastasis and an important prognostic factor in advanced ovarian cancer. We analyzed the publicly available microarray dataset (GSE2109) using binary regression and found that the transforming growth factor (TGF)-beta signaling pathway was activated in omental metastases as compared to primary sites of disease. Immunohistochemical analysis of TGF-beta receptor type 2 and phosphorylated SMAD2 indicated that both were upregulated in omental metastases as compared to primary disease sites. Treatment of the mouse ovarian cancer cell line HM-1 with recombinant TGF-β1 promoted invasiveness, cell motility and cell attachment while these were suppressed by treatment with A-83-01, an inhibitor of the TGF-β signaling pathway. Microarray analysis of HM-1 cells treated with TGF-β1 and/or A-83-01 revealed that A-83-01 efficiently inhibited transcriptional changes that are induced by TGF-β1. Using gene set enrichment analysis, we found that genes upregulated by TGF-β1 in HM-1 cells were also significantly upregulated in omental metastases compared to primary sites in the human ovarian cancer dataset, GSE2109 (false discovery rate (FDR) $q = 0.086$). Therapeutic effects of A-83-01 in a mouse model of peritoneal dissemination were examined. Intraperitoneal injection of A-83-01 (150 μg given three times weekly) significantly improved survival ($p = 0.015$). In summary, these results show that the activated TGF-β signaling pathway in peritoneal metastases is a potential therapeutic target in ovarian cancer.

Ovarian cancer is the most lethal gynecologic cancer in the Western world. Because of its absence of obvious symptoms, the diagnosis of ovarian cancer is often made when the disease is at an advanced stage, resulting in a survival rate of only 20–30%.[1] Peritoneal dissemination is the most frequent route of spread and is the most significant prognostic factor in ovarian cancer.[2] Although many efforts have been made to treat peritoneal dissemination of ovarian cancer, such as debulking surgery and systemic or intraperitoneal chemotherapy, effective eradication of peritoneal dissemination remains challenging. Therefore, there is an urgent need to develop new treatment modalities, especially targeted molecular therapies, through the study of basic biology underlying peritoneal disease spread.

Recently, the roles of the transforming growth factor (TGF)-beta signaling pathway in cancer have been studied in great depth, particularly in the progression of cancer and metastasis.[3,4] In the early phases of cancer development, TGF-β is thought to suppress the proliferation of tumor cells. In the advanced phases, however, TGF-β may promote cancer progression. Inhibition of TGF-β2 production by antisense oligonucleotides (AP12009) is a promising therapeutic strategy against glioma[5] and a phase III clinical trial is currently underway. However, little is known about the role of the TGF-β signaling pathway in ovarian cancer.

In our study, we performed gene expression microarray analysis and revealed that the TGF-β signaling pathway is activated in omental metastases of ovarian cancer. Activated TGF-β signaling promoted metastatic properties of ovarian cancer cells, including adhesion to extracellular matrix, cell motility and invasiveness. In addition, we examined the potential efficacy of A-83-01, a small molecule inhibitor of TGFBR1 kinase activity, in the treatment of ovarian cancer

Key words: ovarian cancer, metastasis, TGF β
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metastases, both *in vitro* and *in vivo*. This report describes for the first time that the activated TGF-β signaling pathway in omental metastases of ovarian cancer is a potential therapeutic target.

Material and Methods

Cell line

The OV2944-HM-1 (HM-1) mouse ovarian cancer cell line, purchased from the RIKEN BioResource Center (Tsukuba, Japan), was cultured as previously described.[6] The culture medium consists of minimum essential medium (MEM) alpha (Invitrogen, Carlsbad) supplemented with 10% heat-inactivated fetal bovine serum (v/v; Biowest, France) and penicillin–streptomycin (100 IU/ml penicillin, 100 μg/ml streptomycin; Nacalai Tesque, Kyoto, Japan). SK-OV-3 human ovarian cancer cell line was purchased from the ATCC (Rockville, MD) and cultured in RPMI1640 (Nikken Biomedical Laboratory, Kyoto, Japan) supplemented with the same reagents as MEM alpha.

Reagents

A-83-01, a small molecule inhibitor of TGF-β receptor type I kinase activity, was purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human TGF-β1 was purchased from Peprotech (Rock Hill, NJ).

Microarray analysis

HM-1 cells were treated with A-83-01 (1 μM) or vehicle dimethyl sulfoxide (DMSO) for 2 hr followed by the addition of TGF-β1 (1 ng/ml) or vehicle phosphate buffered saline (PBS). Four hours later, HM-1 cells were harvested, and RNA was extracted using the RNeasy Mini Kit (Qiagen). Gene expression data were generated at Singapore University as previously described.[7] We used 200 ng total RNA per sample on Affymetrix Mouse ST v1.0 GeneChips containing probes for over 28,000 well-annotated genes.

Microarray datasets were obtained from the Gene Expression Omnibus website (http://www.ncbi.nlm.nih.gov/geo/). Serous ovarian cancer samples (primary; $n = 88$, omental metastasis; $n = 38$) in GSE2109 were used for the analysis. Of these, 15 cases had received chemotherapy prior to the operation. Samples used in the study are listed in Supporting Information Table 1.

Bioinformatics analyses

Normalization. Data with the MAS5 format were used for binary regression analyses, whereas other analyses were performed using robust multi-array average (RMA)-normalized data. *R* version 2.8.2 was used for the normalization.[8]

Genes differentially expressed between control and TGF-β1-treated samples were identified using significance analysis of microarrays (SAM).[9] Gene probes with FDR $q < 0.25$ were selected for further analyses.

Binary regression was performed as previously described.[10–12] This is a tool to determine the probability of gene signature for individual samples to examine pathway activity. Training set had been developed by overexpression of oncogenes (Src, Myc, Ras, β-catenin and E2F3) or addition of cytokines (tumor necrosis factor (TNF)-alpha and TGF-β1) onto cultured human cells. Differentially expressed genes by the treatment were identified and designated as the gene signature. Probabilities of the gene signature can be determined for individual samples in external datasets. In this research, we determined gene signature probabilities for the serous ovarian cancer samples (Figs. 1 and 2*a*).

Gene set enrichment analysis (GSEA) was conducted as previously reported.[13] We used C2 curated gene sets of the MSigDB (http://www.broadinstitute.org/gsea/msigdb/) to determine the enriched pathways in omental metastases of ovarian cancer. Briefly, we performed the GSEA analysis to compare between primary sites ($n = 88$) *versus* omental metastases ($n = 38$) of GSE2109. At first, we applied all the C2 curated gene sets ($n = 1892$) with the following parameters; "Number of permutations = 10" and "Permutation type = phenotype." All other parameters were default. As a result, 657 gene sets showed statistically significant (FDR q value <0.25) enrichment among the omental metastases. To analyze selected gene sets relevant to the TGF-β pathway more accurately, we downloaded several gene sets (that appears in the Results section) from the MSigDB and analyzed individually with the following parameters; "Number of permutations = 1000" and "Permutation type = phenotype". Genes upregulated by TGF-β1 ($n = 173$) in HM-1 cells (Supporting Information Table 2) were also used to generate a gene set for a GSEA analysis. Genes downregulated by TGF-β1 ($n = 12$) were not used for this purpose because this gene set did not meet the gene set size threshold (≥ 15) to run the GSEA software.

An average-linkage hierarchical clustering analysis was performed using Cluster 3.0 available from http://rana.lbl.gov/EisenSoftware.htm. Java TreeView (http://jtreeview.sourceforge.net/) was used to visualize the heat map.

Immunohistochemical staining

Ovarian cancer specimens taken from women who received primary tumor resection at the Department of Gynecology and Obstetrics of Kyoto University Hospital from 2003 through 2006 were used for immunohistochemical staining. Prior written informed consent from all the patients was received, and approval was given by the Kyoto University Graduate School and Faculty of Medicine Ethics Committee. For our study, we used only serous ovarian cancer samples with no prior treatment. Immunohistochemical staining of TGFBR2 was carried out using the streptavidin–biotin–peroxidase method. Briefly, formalin-fixed paraffin-embedded tissue sections were deparaffinized and antigen retrieval was performed in 10 mM sodium citrate buffer (pH 6.0) at 120°C for 5 min. The slides were incubated with primary anti-TGFBR2 antibody at a 1:100 dilution (Abcam, Cambridge, MA) overnight at 4°C, followed by incubation with biotinylated goat anti-rabbit secondary antibodies (Nichirei). For staining of phosphorylated SMAD2 (pSMAD2), anti-pSmad2 antibody (Novus Bio, Littleton, CO) was used at a 1:100

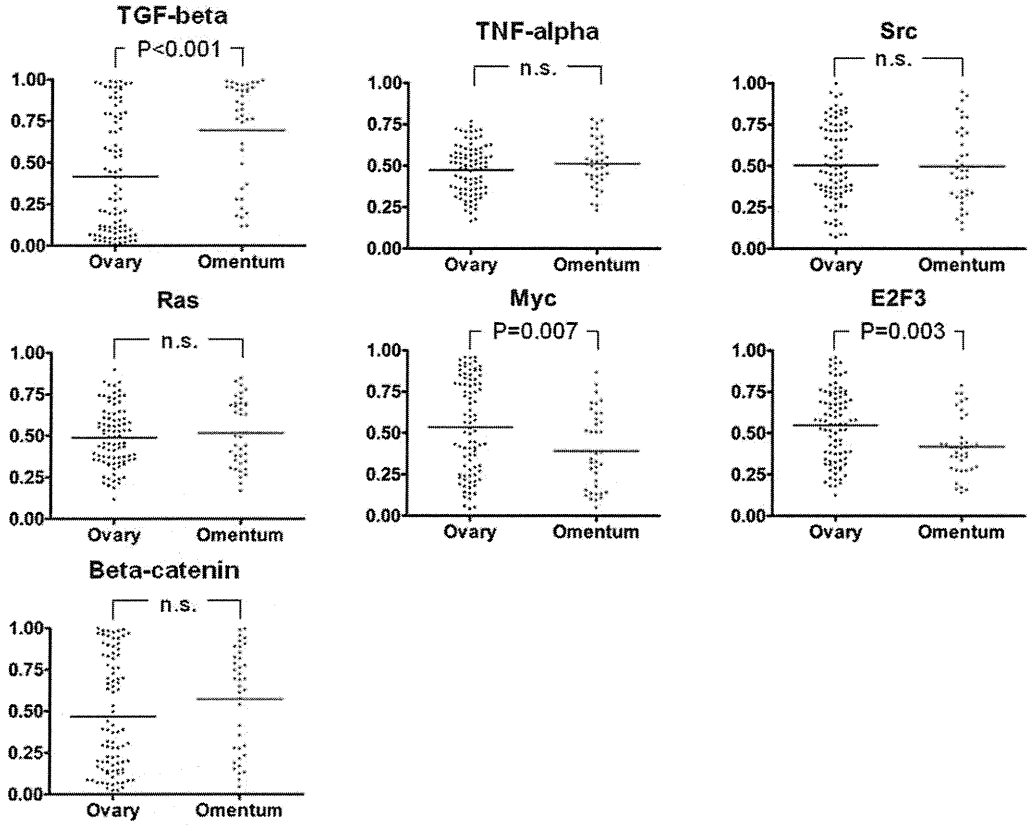

Figure 1. Activities of signaling pathways in primary sites and metastatic sites of ovarian cancer. Activities of seven signaling pathways including TGF-β, TNF-alpha, Src, Ras, Myc, E2F3 and β-catenin were analyzed using binary regression in the GSE2109 dataset. In each scatter diagram, individual dots show the activity of the signaling pathway for 88 primary sites (left) and 38 omental metastases (right) of ovarian cancers. *Y* axis; signature probabilities. n.s.; not significant.

dilution with the same subsequent procedure as TGFBR2. Two independent gynecological pathologists examined the immunohistochemistry slides without any prior information about the clinical history of the patients. TGFBR2 and pSMAD2 expression was evaluated according to staining intensity and was scored as follows: 0, negative, no staining in cancer cells (same or weaker than the cancer stroma); 0.5, weakly positive (staining of the cancer cells is slightly stronger than that of the cancer stroma); 1, positive (staining of the cancer cells is stronger than that of the cancer stroma); 2, strongly positive (staining of the cancer cells is much stronger than that of the cancer stroma).

Western blot analysis

A-83-01 (1 or 10 μM) or vehicle (DMSO) control were added to HM-1 cells followed by TGF-β1 (1 or 10 ng/ml) or vehicle control 30 min later. Cells were harvested 60 min later and lysed in radio-immunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, Waltham, MA) with a protease inhibitor cocktail (EMD, Madison, WI) and a phosphatase inhibitor cocktail (Nacalai Tesque). Protein was quantified using the DC Protein Assay Kit (Bio-Rad, Hercules, CA). Twenty micrograms of sodium dodecyl sulfate (SDS)-treated protein was loaded onto a 10% SDS-PAGE (polyacrylamide gel electrophoresis) gel (Ready Gels J, Bio-Rad). Gels were electroblotted onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad). Nonspecific binding of the antibody was blocked by incubating for 30 min at room temperature in Blocking One-P (Nacalai Tesque). The membranes were incubated overnight at 4°C with antiphosphorylated Smad3 antibody (1:1,000, pSmad3 Rabbit mAb, Immuno-Biology Laboratories, Japan), which reacts with both human and mouse protein. After washing in tris-buffered saline (TBS)-T, the blots were incubated with the appropriate peroxidase-coupled secondary antibody (1:10,000; Anti-rabbit HRP, GE Healthcare Life Sciences, Uppsala, Sweden). An anti-human β-actin antibody (1:5,000; Rabbit mAb, Abcam) were used for the endogenous controls. Specific proteins were detected using ECL Plus Western Blotting Reagent (GE Healthcare Life Sciences).

Cell proliferation assay

HM-1 cells were seeded into a 96-well plate (Asahi Glass, Japan) and were incubated for 18 hr. A-83-01 (1 μM) or vehicle were then added for 12 hr followed by the addition of

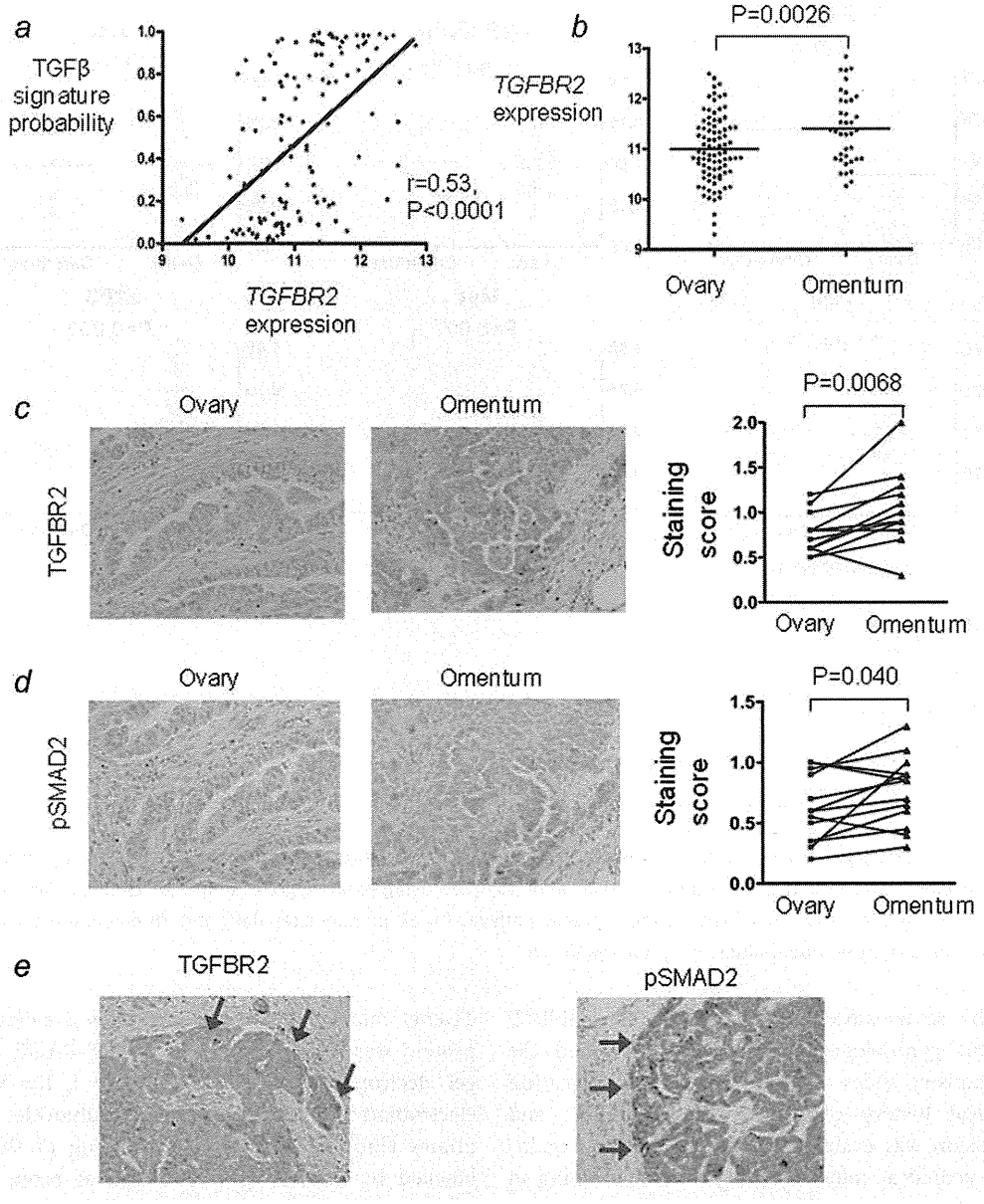

Figure 2. TGFBR2 expression and TGF-β pathway activity in primary ovarian carcinomas and omental metastases. (*a*) Expression of *TGFBR2* (208944_at) is positively correlated with TGF-β signature probabilities in the GSE2109 dataset. TGF-β signature probability for the individual tumor sample was determined as previously described.[12] (*b*) Expression of *TGFBR2* (208944_at) was significantly higher among the omental metastases in the GSE2109 dataset. (*c*) Immunohistochemical expression of TGFBR2. Scatter diagram shows staining scores of the paired samples. (*d*) Immunohistochemical expression and scatter diagram of pSMAD2. The sections of TGFBR2 and pSMAD2 are serial sections. (*e*) Representative figures of TGFBR2 and pSMAD2 expression in the invasive front (red arrows).

TGF-β1 (1 ng/ml) or vehicle for 60 hr. The number of viable cells in each well was examined using the WST-1 assay (Takara Bio) following the manufacturer's instructions.

Cell adhesion assay

HM-1 cells were treated with A-83-01 (1 μM) or vehicle for 12 hr followed by treatment with TGF-β1 (1 ng/ml) or vehicle for 8 hr. Next, 10^5 cells were seeded into each well of collagen IV-coated 96-well plates (Asahi Glass) and incubated for 2 hr. After that, wells were washed twice with PBS, and the number of attached cells was evaluated using the WST-1 assay.

Cell migration assay

HM-1 cells were seeded onto 10 cm tissue culture dishes (Greiner bio-one, Monroe, NC) and incubated until cells were confluent. Next, scratch lines (1000–1200 μm width) were introduced using a plastic tip and A-83-01 (1 μM) or vehicle was added. Six hours later, TGF-β1 (1 ng/ml) or

vehicle was added. The widths of the gaps in the cell monolayers were measured 20 hr after scratch introduction.

Cell invasion assay

HM-1 cells were treated with 1 μM of A-83-01 for 12 hr followed by treatment with 1 ng/ml of TGF-β1 for 8 hr. Next, 10^5 cells were seeded into Boyden chambers with 8.0 μm pore PET membranes (Becton Dickinson, Franklin Lakes, NJ) coated with Matrigel. After 20 hr, membranes of the Boyden chambers were fixed with 99% methanol and stained in hematoxylin. The number of cells that invaded through the membrane were visually counted in five high power fields (200×) and averaged. To confirm the effect of TGF-β1 and A83-01 on human ovarian cancer cell lines, we did invasion assay with SK-OV-3 cells.

ELISA assay of TGF-β1

Ascites were retrieved from the abdominal cavity of mice 10 days after inoculation with 1×10^6 HM-1 cells. The concentration of TGF-β1 in ascites was determined with the Quantikine human TGF-β1 immunoassay kit (R&D Systems, Minneapolis, MN) following manufacturer's instructions. The optical density of each well was read with an Emax microplate reader (Molecular Devices, Silicon Valley, CA).

In vivo experiments

Female B6C3F1 mice used for the *in vivo* studies were purchased from CLEA Japan (Tokyo, Japan) and maintained under specific pathogen-free conditions. All animal experiments were approved by the Kyoto University Animal Research Committee. To evaluate the effect of A-83-01 on the survival of mice bearing peritoneal dissemination, HM-1 cells (1×10^6) were injected into the abdominal cavity *via* the left flank of the mouse. Starting the next day, A-83-01 (150 μg/body) or vehicles (PBS with 0.5% DMSO) were injected into the abdominal cavity three times per week. Mice were euthanized before reaching the moribund state.

Statistical analysis

All statistical analyses were performed using Prism 4.0b. Student's *t*-test was used unless indicated in the text. p value < 0.05 was considered statistically significant.

Results

Identification of the TGF-β activated signaling pathway in the omental metastases of ovarian cancer by gene expression profiling analysis

To investigate signaling pathways that may differ between the primary site and the omental metastases of ovarian cancer, we conducted gene expression microarray analysis using the publically available dataset GSE2109. Based on the previously reported gene signatures of seven signaling pathways (Src, Myc, Ras, TNF-alpha, β-catenin, TGF-β and E2F3),[10–12] we evaluated signature probabilities on behalf of each pathway in this data. Among the seven pathways, only the TGF-β signaling pathway was more active in the omental metastases than the primary sites (Fig. 1, $p < 0.0001$). In contrast, the Myc and E2F3 pathways were more activated in the primary sites ($p < 0.007$ and $p < 0.003$, respectively).

Consistent with the result from the binary regression, GSEA revealed that "TGF_BETA_SIGNALING_PATHWAY" was enriched in the omental metastases (FDR q value = 0.03). Furthermore, the characteristic *in vitro* effects of TGF-β on cancer cells[14–16] such as "CELL_MOTILITY," (FDR $q < 0.001$) "CELL_ADHESION" (FDR $q < 0.001$) and "MATRIX_METALLOPROTEINASES" (FDR $q = 0.006$) were also enriched in the omental metastases (data not shown).

Comparison of TGFBR2 and pSMAD2 expression in paired samples of primary site and omental metastases by immunohistochemistry

Among the core components of the TGF-β signaling pathway, *TGFBR2* (208944_at) showed a significant positive correlation with TGF-β signature probability scores ($r = 0.53$, $p < 0.0001$, Pearson's correlation) (Fig. 2*a*). In addition, *TGFBR2* expression was significantly upregulated in the omental metastases compared to primary sites ($p = 0.0026$; Fig. 2*b*).

We next examined expression of TGFBR2 and phosphorylated SMAD2 (pSMAD2) in ovarian cancer tissue samples by immunohistochemistry. We used samples for which both primary and omental metastasis sites were available ($n = 14$), and we compared their expression using paired analysis. Both TGFBR2 and pSMAD2 were upregulated in the omental metastases ($p = 0.0068$ and $p = 0.040$, respectively, Figs. 2*c* and 2*d*). In addition, among the cancer tissues, expression of both TGFBR2 and pSMAD2 tended to be stronger at the invasive front and in the small cancer nests rather than the center of tumor nodules (Fig. 2*e*).

Effect of TGF-β1 and the TGF-β pathway inhibitor A-83-01 on the in vitro metastatic properties of ovarian cancer cells

The *in vitro* effects of recombinant TGF-β1 or the TGF-β pathway inhibitor A-83-01 were examined using the HM-1 mouse ovarian cancer cell line.[6] First, we examined the expression of phosphorylated Smad3 (pSmad3) by Western blotting. As expected, expression of pSmad3 was increased by the addition of TGF-β1, and the effects of TGF-β1 were inhibited by A-83-01 (Fig. 3).

Addition of TGF-β1 or A-83-01 did not alter proliferation of HM-1 cells (Fig. 4*a*). Thus, we next examined the influence of TGF-β1 or A-83-01 on cell motility, adhesion and invasion. TGF-β1 increased cell motility, adhesion and invasion, while A-83-01 decreased these behaviors (Figs. 4*b*, *c* and *d*). When only A-83-01 was added, cell motility, adhesion and invasion were decreased (Figs. 4*b*, *c* and *d*). This appears to be a consequence of inhibition of autocrine/paracrine TGF-β signaling in HM-1 cells because the concentration of TGF-β1 in the culture medium (1,260 ± 17 pg/ml) increased after HM-1 cells were cultured for 18 hr (3,395 ± 15 pg/ml; data not shown).

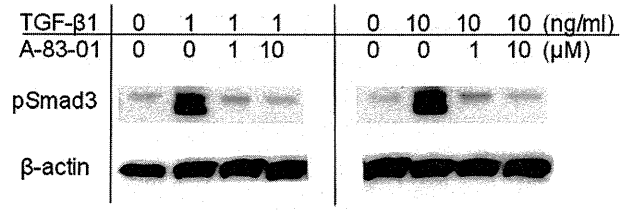

Figure 3. Transcriptional changes of HM-1 cells with TGF-β1 and/or A-83-01. Western blotting for pSMAD3 of HM-1 cells in reaction to TGF-β1 (1 or 10 ng/ml) and/or A-83-01(1 or 10 μM).

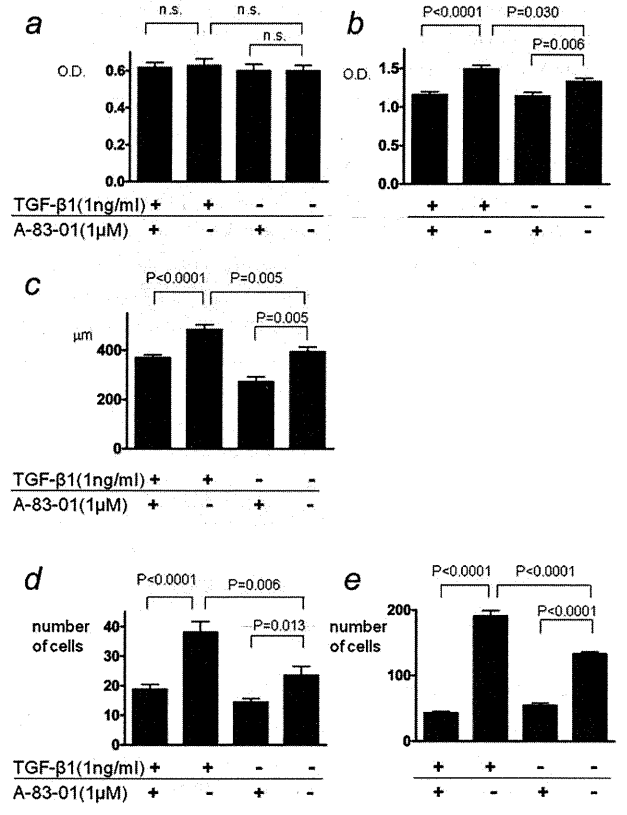

Figure 4. *In vitro* treatment of HM-1 cells and SK-OV-3 cells with TGF-β1 and/or A-83-01. (*a*) WST-1 assay of HM-1 cells to analyze proliferation of cells 60 hr after treatment ($n = 12$ each). (*b*) WST-1 assay of HM-1 cells to analyze the adhesion of cells onto collagen type IV-coated dishes, 150 min post-treatment ($n = 20$ each). (*c*) Wound healing assay of HM-1 cells to analyze cell motility ($n = 12$ each). The *y* axis indicates the distance of migration 20 hr post-treatment. (*d*) Boyden chamber assay of HM-1 cells to analyze cell invasion. Invaded cells were counted 20 hr post-treatment ($n = 12$ each). *Y* axis indicates the average of invading cell numbers in high power fields (200×). (*e*) Boyden chamber assay of SK-OV-3 cells to analyze cell invasion.

As to invasion assay, SK-OV-3 cells, human ovarian cancer cells, showed same tendency as HM-1 cells with addition of TGF-β1 or A-83-01 (Fig. 4*e*).

Identification of genes that are regulated by TGF-β

We next conducted microarray analysis using HM-1 cells to compare gene expression among vehicle ($n = 3$), TGF-β1 ($n = 3$), TGF-β1 + A-83-01 ($n = 3$) or A-83-01 treatments ($n = 1$). Differentially expressed genes between vehicle and TGF-β1-treated samples were identified using SAM. A cut-off of FDR $q < 0.25$ identified 241 probes, including 218 probes (173 genes) upregulated and 23 probes (12 genes) downregulated by TGF-β1 (Supporting Information Table 1).

An average-linkage hierarchical analysis of the 10 HM-1 samples using the above 241 probes was performed. As expected, these genes were found to distinguish TGF-β1-treated samples from the other samples (Fig. 5). The similarity of the TGF-β1 + A-83-01 group with vehicle and not the TGF-β1 group indicates that A-83-01 efficiently reversed gene expression changes induced by TGF-β1.

The 173 upregulated genes were used in a GSEA analysis to determine if they were differentially expressed between primary sites and the omental metastases of ovarian cancer in the GSE2109 dataset. These genes were significantly enriched among the omental metastases (FDR *q* value = 0.086 <0.25).

Therapeutic effects of A-83-01 in a mouse model of peritoneal dissemination of ovarian cancer

We first examined side effects of A-83-01 in B6C3F1 mice. Peritoneal injection of A-83-01 twice a week for 4 weeks at doses of 50, 150 and 500 μg did not influence body weight or neurobehavioral appearances. No change was found in the histolopathological appearance of lung, heart, liver or kidney (data not shown).

We then investigated the therapeutic potential of A-83-01 in a mouse model of peritoneal dissemination of ovarian cancer. Intraperitoneal injection of 1×10^6 HM-1 cells into B6C3F1 mice led to ascites accumulation with diffuse disseminated tumors on the peritoneum within 2 weeks (Fig. 6*a*). The ascites contained abundant TGF-β (26.993 ± 0.068 ng/ml, data not shown). A-83-01 was injected into the peritoneal cavity (150 μg/mouse × 3 times/week) starting 1 day postinjection of HM-1 cells. Formation of ascites tended to be slower in the A-83-01-treated group (Supporting Information Fig. 1), and A-83-01 significantly improved survival of the mice (Fig. 6*b*, $p = 0.0148$).

Discussion

Ovarian cancer is the most lethal malignancy among the gynecological cancers because most cases are diagnosed only after the disease has reached an advanced stage. Peritoneal dissemination, which frequently involves the omentum, is the most typical mode of spread of ovarian cancer.[2] To clarify the molecular mechanisms underlying the metastasis of ovarian cancer, we compared the primary site and omental metastases of ovarian cancer. We have recently used bioinformatics approaches such as binary regression to elucidate

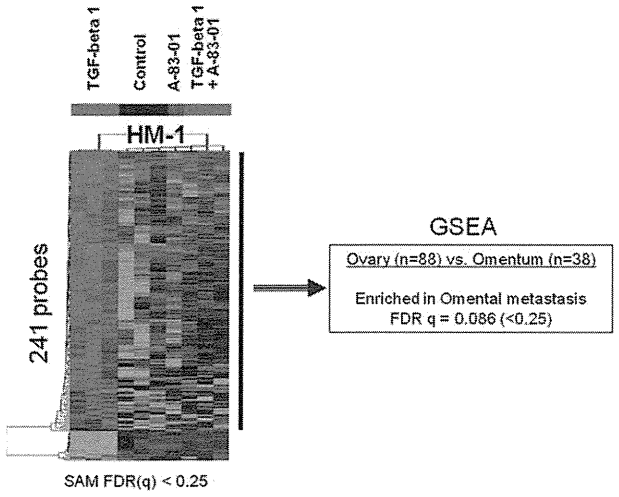

Figure 5. Identification of genes regulated by TGF-β1 in HM-1 cells. Microarray analysis of HM-1 cells for the four treatment groups; control ($n = 3$), TGF-β1 ($n = 3$), TGF-β1 + A-83-01 ($n = 3$) and A-83-01 only ($n = 1$). Average-linkage hierarchical analysis was performed using the 241 differentially expressed probes (FDR $q < 0.25$) between the control and TGF-β1-treated cells. Upregulated genes were used for GSEA analysis to examine relevance with the clinical cancer dataset GSE2109.

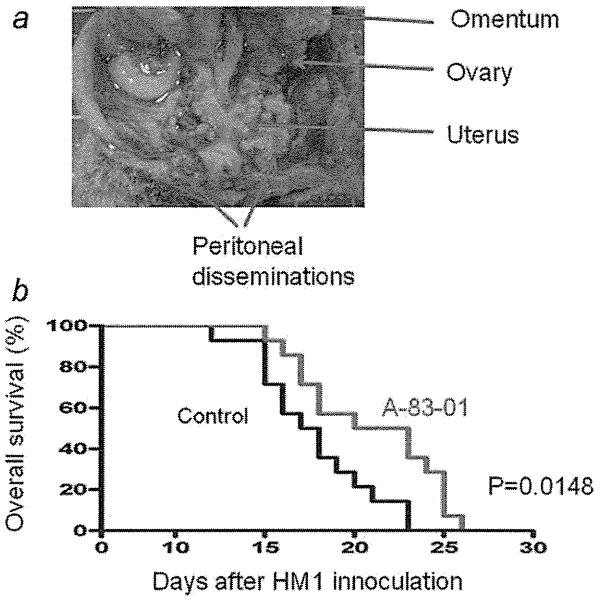

Figure 6. Mouse model of intraperitoneal dissemination using HM-1 cells and treatment with A8301. (*a*) Intraperitoneal findings after 10 days of HM-1 cells inoculation. Disseminated nodules can be seen on the peritoneum and abdominal organs, especially omentum, mesentery, uterus and ovary. (*b*) Survival curves of the A-83-01 treated group and the control group ($n = 14$ each).

activated signaling pathways in cancer.[10–12,17,18] In our study, we found that the TGF-β pathway activity was upregulated in the omental metastases compared to primary sites (Fig. 1). GSEA also demonstrated enrichment of gene sets relevant to the TGF-β signaling pathway. Based on the results from the computational screening, we further examined expression of pSMAD2, a representative molecule indicating activated TGF-β signaling.[19] Immunohistochemical analysis revealed significant upregulation of pSMAD2 expression in the omental metastases (Fig. 2*b*). These results demonstrated that the TGF-β signaling pathway is activated in ovarian cancer metastases. Furthermore, pSMAD2 expression was stronger in the invasive front of cancer nests (Fig. 2*d*). Localized TGF-β signaling within cancer tissue may be attributed to the inherent heterogeneity of the tumor. Here, we found higher expression of pSMAD2 in invasive fronts and small cancer nests, and this would result in higher expression overall in metastases because invasive fronts tended to be more abundant in omental metastasis than in primary sites (data not shown). Nonetheless, activation of the TGF-β signaling pathway suggests a role in the metastatic process of ovarian cancer.

Expression of *TGFBR2*, a core component of the TGF-β signaling pathway, showed a significant positive correlation with TGF-β signature probability scores (Fig. 2*a*), and TGFBR2 was upregulated in metastases similar to pSMAD2 (Figs. 2*c*, 2*e*). Thus, transcriptional regulation of *TGFBR2* could play a crucial role in the regulation of TGF-β signaling. Although TGF-β signaling leads to cell cycle arrest and/or apoptosis of normal epithelial cells, this "tumor-suppressive function" of TGF-β signaling is inhibited through genetic or epigenetic changes in pathway components in virtually all types of cancers.[20] Lynch et al.[21] reported that in ovarian cancer, expression of TGFBR2 is lost by mutations in the coding regions of the *TGFBR2* gene. However, there have been several reports that challenge this observation. Another group showed that the frequency of *TGFBR2* mutation is quite rare in ovarian cancer.[22] In most cases of ovarian cancer, addition of TGF-β to primary cancer cells leads to activation of internal signals.[23,24] In our study, given the specific expression of pSMAD2 (Fig. 2*d*) in tumor cells, loss of internal signaling by receptor mutations is unlikely. Epigenetic regulation, however, could be one possible mechanism that alters transcriptional regulation of *TGFBR2*. We recently found that promoter DNA methylation of the *TGFBR2* gene is often found in ovarian cancer and its status negatively correlates with *TGFBR2* expression (submitted for publication). However, further studies are necessary to clarify the precise mechanisms of activation of the TGF-β signaling pathway in omental metastases.

Next, to reveal the functional role of the TGF-β signaling pathway in ovarian cancer, we conducted *in vitro* assays using the HM-1 mouse ovarian cancer cell line.[6] In our preliminary experiments, HM-1 cells expressed *Muc16* (data not shown), an ortholog of human *MUC16*, which encodes CA125, a specific biomarker of human epithelial ovarian cancer. The addition of TGF-β1 increased expression of pSmad3 in HM-1 cells (Fig. 3), indicating that the signaling pathway is not disrupted at the receptor level. In *in vitro* experiments,

addition of TGF-β1 increased cell motility, invasiveness and attachment of HM-1 cells (Figs. 4*b*, *c* and *d*), and these effects were inhibited by the addition of A-83-01. We also conducted similar experiment using SK-OV-3, human serous ovarian cancer cell line, which showed the same tendency as HM-1 cells in reaction to TGF-β1 and A-83-01. These effects of TGF-β1 might promote detached cancer cells to attach to the extracellular matrix and promote invasion into the peritoneal membrane, resulting in peritoneal dissemination. A-83-01 is likely to block TGF-β1-induced metastatic properties efficiently through disrupting kinase activity of TGF-β type 1receptor, although its inhibitory effects through other type 1 receptor of TGF-β superfamily such as ALK4 and ALK7 might not be excluded.[25] This prominent inhibitory effect of A-83-01 suggests the potential utility of A-83-01 in the treatment of ovarian cancer. In contrast, TGF-β1 or A-83-01 did not affect the proliferation of HM-1 cells (Fig. 4*a*), indicating that the "tumor suppressive" activity is not functional, as in many cancer cells. Interestingly, GSEA analysis to clarify the differences between primary sites and omental metastases of ovarian cancers indicated that gene sets relevant to cell motility, adhesion and invasion were enriched among the omental metastases, suggesting an *in vivo* role of the TGF-β pathway in ovarian cancer.

We next examined transcriptional changes caused by TGF-β1 and/or A-83-01 in HM-1 cells and found that A-83-01 reversed the effects of TGF-β1 (Fig. 3). From GSEA analysis, genes upregulated by TGF-β1 in HM-1 cells were highly expressed in omental metastases of ovarian cancer. These results indicated that the TGF-β signaling pathway in HM-1 cells bears relevance to omental metastasis in human ovarian cancer. Genes upregulated by TGF-β1 in HM-1 cells included several "metastasis promoter genes", such as acylglycerol kinase (*AGK*), granulin (*GRN*), heparanase (*HPSE*), oncostatin M (*OSM*), *slingshot homolog2 (SSH2)* and TGF-β-induced (*TGFBI*) (Supporting Information Table 1). AGK increases formation and secretion of lipoprotein(a) LPA, resulting in increased migration of prostate cancer cells.[26] HPSE increases metastatic potential by remodeling extracellular matrix in many types of cancers.[27] GRN increases the motility and invasion of ovarian cancer cells[28] and in hepatocellular carcinoma.[29] OSM increases the invasive capacity of breast cancer cells.[30] SSH2-mediated dephosphorylation of cofilin/ADF (actin depolymerizing factor) increases the migration and invasion of hepatoma cells.[31] TGFBI promotes metastasis of colon cancer cells by accelerating cell extravasation.[32] Although further investigation is necessary, increased metastatic properties resulting from TGF-β signaling in ovarian cancer may be attributed to upregulation of multiple metastasis-promoting genes.

Finally, we analyzed the *in vivo* therapeutic effects of A-83-01. As A-83-01 has never been used for *in vivo* experiments, we first examined side effects of A-83-01 in mice; no significant side effects were observed as the previous reports with the other TGF-β inhibitors.[14,15] It is known that considerable amounts of TGF-β1 exist in the ascitic fluid of ovarian cancer patients.[33] Similarly, the concentrations of TGF-β1 were high in the ascitic fluid associated with peritoneal dissemination of HM-1. Therefore, this mouse model is appropriate to examine the *in vivo* therapeutic effects of A-83-01. Accumulation of ascitic fluid in A-83-01-treated mice tended to be slower than the control group (Supporting Information Fig. 1). In addition, A-83-01 significantly improved survival (Fig. 6*b*). Recently, inhibition of the TGF-β pathway has been shown to be a promising treatment modality against cancers. AP12009, an antisense oligonucleotide against TGF-β2, was found to be very effective against recurrent glioma in a phase I/II clinical trial,[5] and a phase III clinical trial is now underway. Efficacy of TGFBR kinase inhibitors, such as A-77, Ki26894 and LY2109761 was demonstrated in preclinical studies of gastric cancer, breast cancer and pancreatic cancer.[14,15,34] Although there may be a risk of carcinogenesis[20] with these drugs, this has never been reported in clinical and preclinical studies for TGF-β inhibitors. Therefore, A-83-01 may be a useful drug in the treatment of advanced ovarian cancer.

In summary, starting from gene expression profiling analysis, we found that the TGF-β signaling pathway is activated in omental metastases of ovarian cancers. We then demonstrated the *in vitro* and *in vivo* efficacy of A-83-01, a TGFBR1 kinase inhibitor. This report shows for the first time that inhibition of the TGF-β signaling pathway by a small molecule is a promising strategy in the treatment of ovarian cancer.

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Differential effects of inhibition of bone morphogenic protein (BMP) signalling on T-cell activation and differentiation

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Bone morphogenetic proteins (BMPs) are involved in patterning and cellular fate in various organs including the thymus. However, the redundancy of BMPs and their receptors have made it difficult to analyse their physiological roles. Here, we investigated the role of BMP signalling in peripheral CD4$^+$ T cells by analysing the effects of an inhibitor of BMP signalling, dorsomorphin. Dorsomorphin suppressed phosphorylation of SMAD1/5/8, suggesting that BMP signalling naturally occurs in T cells. At high doses, dorsomorphin suppressed proliferation of T cells in a dose-dependent manner, inducing G1 arrest. Also, dorsomorphin suppressed Th17 and induced Treg-cell differentiation, while preserving Th2 differentiation. Dorsomorphin efficiently suppressed IL-2 production even at low doses in mouse CD4$^+$ T cells, suggesting that the BMP-Smad signalling physiologically regulates IL-2 transcription in these cells. In addition, recombinant BMP2 induced a dose-dependent multiphasic pattern of IL-2 production, while Noggin suppressed IL-2 production at higher doses in Jurkat cells. Notably, BMP signalling controlled the phosphorylation of RUNX1, revealing the molecular nature of its effect. Collectively, we describe multiple effects of dorsomorphin and Noggin on T-cell activation and differentiation, demonstrating a physiological role for BMP signalling in these processes.

Key words: Bone morphogenic protein · CD4$^+$ T cells · IL-2 · Morphogen · T-cell activation

Supporting Information available online

Introduction

T cells are continuously generated in the thymus, and differentiate into more mature T cells in the periphery throughout life. Thus, not surprisingly, many mechanisms involved in embryogenesis are used in thymocytes and T cells. Bone morphogenetic proteins (BMPs) have important roles in embryogenesis and organogenesis of various tissues including the development of the thymus [1]. It has been reported that BMP2 and BMP4 and their extracellular inhibitors, Noggin and Chordin, are expressed in the thymus, and that BMP4 inhibits the transition from CD4$^-$CD8$^-$ double negative (DN) to CD4$^+$CD8$^+$ double positive (DP) and

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arrests cells at the $CD25^{-}CD44^{+}$ and $CD25^{+}CD44^{-}$ DN stages [2, 3]. On the other hand, inhibition of BMP2/4 signalling by Noggin promotes and accelerates thymocyte differentiation, increasing the proportions of $CD44^{-}CD25^{-}$ DN thymocytes and DP thymocytes [2, 3]. In the periphery, the developmental or differentiation processes of T cells are studied as "T-cell activation and differentiation". Thus, the roles of BMP can be reasonably addressed in some aspects of these processes. Recently, BMPs have been suggested to have some roles in peripheral T-cell proliferation, although their physiological relevance and mechanisms are still unclear [4].

BMPs are made up of more than 20 distinct BMP subunits, and belong to the larger transforming growth factor-beta (TGF-β ligand family [5]. BMP forms heterodimers or homodimers, which make heterotetrameric complexes with type I and II BMP receptors (BMPRs), and transduce signals via the complex. BMP ligands are recognized by the pairs of type II BMPR or Activin type II receptor (ActRIIa and ActRIIb) with various BMP type I receptors (ALK1, ALK2, ALK3, and ALK6). When engaged by ligand, type II BMPR phosphorylates type I BMPR, which triggers phosphorylation of BMP-responsive receptor-regulated SMADs (R-SMADs, namely, SMAD1, SMAD5 and SMAD8). R-SMADs form a complex with common-partner SMADs (Co- SMADs: SMAD4) and translocate into the nucleus, where the complex regulates the transcription of its target genes [6]. It is known that the SMAD complex interacts with the transcription factor RUNX2, which is a major target of BMP signalling for the differentiation of osteoblasts [7]. In hematopoietic stem cells, BMP4/SMAD signalling is reported to regulate RUNX1 activity [8]. Preceding studies indicate that BMP activates not only the SMAD pathway but also other pathways such as mitogen-activated protein kinase (MAPK) family of molecules including ERK1/2 and p38 [9].

Considering the number of combinations of BMP ligands and receptors, we attempted to address the possible roles of BMP signalling in T-cell activation and differentiation by inhibiting a wide range of BMP signalling using a small molecule inhibitor of type I BMPRs, dorsomorphin (DM) [10]. In this study, we analysed the perturbation effects of DM in $CD4^{+}$ T cells, in order to identify functional consequences of inhibition of BMP signalling in T cells. We found that DM differentially affected the processes of T-cell activation in a unique manner. Presumably, some, if not all, of the mechanisms affected by DM-treatment should include those truly regulated by BMP signalling in T cells.

Results

DM inhibits BMP-SMAD1/5/8 signalling in Jurkat cells

We investigated the role of the BMP signalling pathway by analysing the effects of its inhibitor. A small molecule compound, DM, is reported to selectively decrease the level of SMAD1/5/8 phosphorylation by inhibiting the kinase activity of BMP type I receptor [11]. We confirmed this specific effect of DM on SMAD1/5/8 phosphorylation in Jurkat cells (Fig. 1). DM decreased the level of phosphorylation of SMAD1/5/8 (p-SMAD1/5/8) in both BMP2-treated and non-treated Jurkat cells (Fig. 1A and B). Although the signal/noise ratio of the detection of p-SMAD1/5/8 by western blot using Jurkat were not large, DM clearly inhibited phosphorylation of SMAD1/5/8 in Jurkat cells in a dose-dependent manner, and the half maximal inhibitory concentration (IC_{50}) was ~0.31 μM, which was comparable to the preceding study (Fig. 1C and D, [11]). On the other hand, dorsomophin did not inhibit phosphorylation of SMAD2, which mediates TGF-β signalling (Fig. 1C and D). In addition, BMP2 increased phosphorylation of Smad1/5/8 in mouse $CD4^{+}$ T cells, whereas DM inhibited it in both BMP2-treated and non-treated T cells (Supporting Information Fig. 1). Thus, DM is a specific inhibitor of BMP-SMAD signalling in T cells, as the previous report showed using pulmonary artery smooth muscle cells [11].

DM inhibits T-cell proliferation

Next, we addressed functional consequences of the inhibition of BMP-SMAD signalling by DM. Jurkat or mouse $CD4^{+}$ T cells were cultured with titrated doses of DM (0.1–20 μM) under stimulation with anti-CD3 and -CD28 antibodies for 60 h. Thymidine incorporation assay revealed that DM suppressed the proliferation of both Jurkat and mouse $CD4^{+}$ T cells in a dose-dependent manner (IC_{50} ~2.43 μM, 1.40 μM, respectively, Fig. 2A and B). As expected from this result, DM inhibited cell division of mouse $CD4^{+}$ $CD25^{-}$ naïve T cells by the carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution assay. In the CFSE dilution assay, almost all cells were dead when the concentration of DM was higher than 10 μM (Supporting Information Fig. 2A), presumably because of the synergistic toxicities of CFSE and DM (Fig. 3). DM clearly inhibited cell division by the CFSE dilution assay at the concentrations above 1 μM (Fig. 2C), which was compatible with the results of thymidine incorporation. The Proliferation Index (the average number of cell divisions that a cell in the original population has undergone) showed a dose-dependent inhibition of cell division, while mean fluorescence intensity (MFI) and Division Index (the average number of cell divisions that the responding cells underwent) showed a mild increase of cell division at the low concentration of DM (Fig. 2C). This result suggests that low concentrations of DM can mildly promote the proliferation of dividing T cells. This issue will be addressed below.

DM induces G0/G1 arrest in T cells

In order to analyse more precisely how DM suppresses the proliferation of T cells, we performed cell cycle analysis of DM-treated cells. Jurkat cells were stimulated by anti-CD3 and -CD28 antibodies with DM or DMSO as a control. Cultured cells were analysed 2 days after stimulation by flow cytometry using bromodeoxyuridine (BrdU) label and 7-Amino-actinomycin D (7AAD) staining. The percentages of sub-G1 positive cells were comparable between control and DM-treated cells (6.8 versus

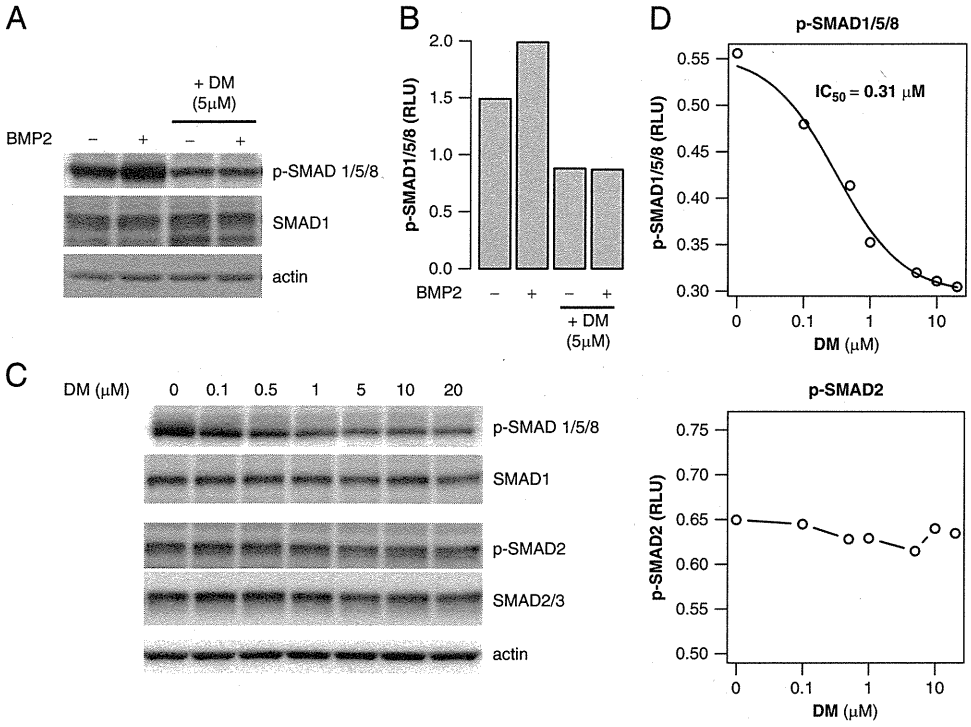

Figure 1. The effect of DM on the phosphorylation of SMAD1/5/8. (A) Western blotting of phosphorylated SMAD1/5/8 (p-SMAD1/5/8) in Jurkat cells cultured with or without BMP2 in the presence of DM or DMSO as a control. Cells were cultured for 30 min with indicated conditions and stimulated with anti-CD3 and -CD28 antibodies for 1 h. (B) Densitometric analysis of p-SMAD1/5/8 in (A). Intensities of p-SMAD1/5/8 were normalised to those of total SMAD1. (C) Western blotting of p-SMAD1/5/8, SMAD1, p-SMAD2, and SMAD2/3 in Jurkat cells cultured with titrated doses of DM. (D) Densitometric analysis of p-SMAD1/5/8 and p-SMAD2 in (C). Intensities of p-SMAD1/5/8 (left) and p-SMAD2 (right) were normalized to those of total SMAD1 and SMAD2/3 respectively. Data were regressed to a four-parameter log-logistic function in p-SMAD1/5/8. Actin was used for a loading control (A, C). All figures are representative of three independent experiments.

7.2%), suggesting that obvious apoptosis or necrosis did not occur in DM-treated cells at this time point (Fig. 3A). The percentage of G0/G1 cells was increased in DM-treated cells compared with control cells (67.5 versus 57.3%, Fig. 3A). On the other hand, the percentage of S-phase cells was decreased in DM-treated cells (9.3 versus 17.6%, Fig. 3A).

The cyclin-dependent kinase inhibitor $p27^{kip1}$ is known to inhibit CDK2 and the G1-S transition, and previous studies indicate that the protein level of $p27^{kip1}$ is increased in anergic T cells [12]. Western blotting of lysates from Jurkat cells cultured with DM showed that DM accumulated $p27^{kip1}$ in Jurkat cells (Fig. 3B). These results indicate that DM inhibits the G1-S transition.

Flow cytometric evaluation of apoptosis using Annexin V and 7AAD staining confirmed that DM-treated, mouse $CD4^+CD25^-$ naïve T cells were not obviously apoptotic (Fig. 3C and D). In the later days of cell culture, control cells were more apoptotic (Fig. 3C and D), suggesting that activation-induced cell death occurs more in control cells.

CD25 expression upon TCR stimulation is attenuated by DM

The results of flow cytometric evaluation of apoptosis suggested that T-cell activation is suppressed in DM-treated T cells. To address this issue, we analysed the flow cytometric features of activated T cells including the expression of the activation marker of T cells, CD25 (IL-2 receptor α chain), upon stimulation. $CD4^+$ $CD25^-$ T cells were stimulated by anti-CD3 and CD28 with DM (5 μM) or DMSO as a control. Both DM-treated cells and control cells increased FSC and SSC (Fig. 4). CD25 expression occurred both in DM-treated and control cells on both days 1 and 4, although the intensities of CD25 expression were lower in DM-treated T cells (MFI: d1, 600 versus 819; d4, 657 versus 1107, Fig. 4). These results suggest that T-cell activation occurs but is partially suppressed by DM.

DM differentially affects helper and regulatory T-cell differentiation in a unique manner

Next, we analysed DM-induced perturbation of differentiation of Th1, Th2, Th17, and induced regulatory T cells (iTreg cells) in vitro. Mouse $CD4^+CD25^-$ naïve T cells were cultured with DM (4 μM) under the Th/Treg polarizing conditions, and their cytokine production and differentiation status were analysed on days 2, 4, and 8 by flow cytometric analysis of intracellular cytokines or Foxp3. DM significantly inhibited the differentiation of Th17 and iTreg cells, while relatively unaffecting the percentages of differentiated Th2 cells at the early stages of cell culture (Fig. 5A

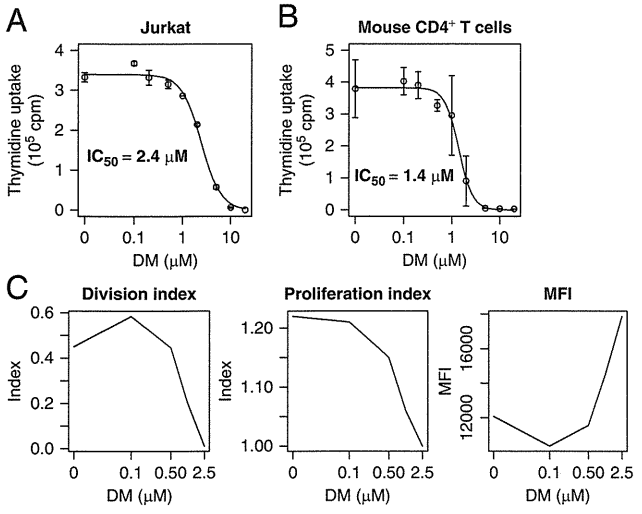

Figure 2. The effects of DM on the proliferation of mouse T cells and Jurkat cells. (A, B) Proliferation of (A) Jurkat or (B) mouse $CD4^+$ T cells cultured with titrated doses of DM was determined by thymidine incorporation. Freshly isolated $CD4^+$ T cells were cultured for 72 h with anti-CD3 and -CD28 antibodies. Data were regressed to a four-parameter log-logistic function. Data are shown as mean±SD of triplicates. (C) Proliferation of T cells was also measured by CFSE dilution assay. Mouse $CD4^+$ $CD25^-$ naïve T cells were cultured with titrated doses of DM, and stimulated by anti-CD3 antibody with T-cell-depleted splenic APCs. Flow cytometric analysis of cultured cells was performed 48 h after stimulation to analyse the intensity of CFSE. The Division Index (the average number of cell divisions that the responding cells underwent), Proliferation Index (the average number of cell divisions that a cell in the original population has undergone) and mean-fluorescence intensity (MFI) of CFSE-stained T cells are shown. See Supporting Information Fig. 1A for gating strategies and density plots for CFSE dilution. All figures are representative of three independent experiments.

and B). Th17 cell differentiation seemed to be continuously suppressed throughout the culture. Interestingly, exogenous IL-2 did not reverse DM-induced effects (Fig. 5A and B). These results indicate that DM affects not only *IL2* transcription (see below) but also other processes that regulate Th/reg differentiation and are independent of IL-2 signalling.

The expressions of the lineage-specific transcription factors of Th cells were analysed by qPCR. DM markedly suppressed the expression of *Tbx21* and *Rorc*, the lineage-specific transcription factors of Th1 and Th17 cells respectively, but did not suppress the expression of *Gata3*, the lineage-specific transcription factor of Th2 cells (Fig. 5C).

Transcriptomic characteristics of DM-treated T cells

In order to obtain the bigger picture of the effects of DM in T-cell activation, transcriptomic analysis was performed using mouse primary $CD25^-CD4^+$ T cells with either DM (4 μM) or vehicle in the presence or absence of stimulation by anti-CD3 and -CD28 antibodies. A moderated *t*-statistic identified 8259 genes as differentially expressed genes in DM-treated T cells (Supporting Information Fig. 3A). More than a third of the genes that were regulated by stimulation were not significantly regulated by DM (Supporting Information Fig. 3A). In contrast, no differentially expressed genes were identified between DM-treated, unstimulated and stimulated T cells, suggesting that DM-treated cells were hyporesponsive upon activation (Supporting Information Fig. 3A and B). Interestingly, however, the expressions of nearly half of the genes that were significantly regulated by DM were not significantly changed by stimulation only (Supporting Information Fig. 3A). Thus, DM seems to induce some unique and specific effects in T cells independent from the general activation process. To characterise these, we performed a pathway analysis [13]. The mechanisms that were downregulated by DM included TCR signalling pathway, helper T-cell surface molecules, and apoptosis-related mechanisms, which are compatible with findings in Figs. 3 and 4 (Supporting Information Fig. 3C). On the other hand, the mechanisms that were upregulated by DM included IL-6 and p38 MAPK singalling pathways. As expected, the mechanisms that were downregulated by the effects of stimulation considering the effects of DM (statistical interaction between DM and stimulation) included chemokines and cytokines, further confirming the results in Figs. 3 and 6. On the other hand, MAPK signalling pathway was identified in the mechanisms that were upregulated in the interaction of stimulation and DM (Supporting Information Fig. 3D). Assume that p38 MAPK pathway is related to BMP-SMAD pathway [9], the apparent increase in the transcripts that were related to this pathway may be considered as a compensatory feedback mechanism. If we assume this relationship between the inhibition of signalling and the compensatory feedback mechanism in transcripts, the observation that TCR signaling pathway was found in the downregulated mechanisms in DM-treated T cells (Supporting Information Fig. 3C) rather supports that the effects of DM is not a simple consequence of the inhibition of TCR signalling pathway.

A complex effect of DM on IL-2 production of T cells

To further address how DM affects T-cell function, we analysed the perturbation of IL-2 production and T-cell differentiation by DM. We focused on IL-2 production for the following reasons. First, IL-2 production is the crucial mechanism of T-cell activation. Second, *IL2* transcription is known to be regulated by several key transcription factors, one of which is AML1/RUNX1, a well-established downstream target of BMP signalling in hematopoietic lineage cells [8, 14, 15].

We found that DM inhibited IL-2 production of Jurkat and mouse $CD4^+$ T cells in a dose-dependent manner (Fig. 6A). Interestingly, IC_{50} of DM for inhibiting the IL-2 production was smaller in mouse $CD4^+$ T cells (~0.73 μM) than in Jurkat cells (~4.2 μM). Quantitative real-time PCR (qPCR) analysis of IL-2 mRNA revealed that DM markedly decreased *IL2* transcript in Jurkat cells at as early as 3 h after stimulation at the concentration of IC_{50} (Fig. 6B).

Interestingly, the dose–response curve of IL-2 production upon TCR stimulation consistently showed a fluctuation at low

