VL	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4	(G4S)3
Clone 1	RHLLTQSH KFLSTSVG NRVSITC	KASQDVST AVA	WYQQKPG QSPKLLIY	SASY RYA	GVPDRFTGSGSGTDFT FTISSVQAEDLAVYYC	QQHYS TPPT	FGGGTK LEIER	GGGGSGG GGSGGGG S
Clone 2	DILLNQSQ KFMSTSVG DRVSVSC	KASQDVDT DVA	WYQQKPG QSPKALIY	SASY RYS	GVPDRFTGSGSGTDFT LTISNVQSEDLAEYFC	QQYNS YPYT	FGGGTK LEIKR	GGGGSGG GGSGGGG S
Clone 3	DILMYQSQ KFMSTSVG DRVSVTC	KASQNVGT NVA	WYQQKPG QSPKALIY	SASY RYS	GVPDRFTGSGSGTDFT LTISNVQSEDLAEYFC	QQYNS YPYT	FGGGTK LEMKR	GGGGSGG GGSGGGG S
Clone 4	DILLTQSPS SLSVSAGEK VTMSC	KSSQSLLNS GNQKNYLA	WYQQKPG QPPKLLIY	GAST RES	GVPDRFTGSGSGTDFT LTISSVQAEDLAVYYC	QNDH SYPLT	FGAGTK LELKR	GGGGSGG GGSGGGG S
Clone 5	DILLNQSQ KFMSTSVG DRVSVSC	KASQDVDT DVA	WYQQKPG QSPALIY	SASY RYS	GVPDRFTGSGSGTDFT LTISNVQSEDLAEYFC	QQYNS YPYT	FGGGTK LEIKR	GGGGSGG GGSGGGG S
Clone 6	DIVITQSHK FMSTSVGD RVSITC	KASQDVGT AVA	WYQQKPG QSPKLLIY	SASY RYT	GVPDRFTGSGSGTDFT FTISSVQAEDLAVYYC	QQHYS TPYT	FGGGTK LEIKR	GGGGSGG GGSGGGG S

VH	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4	FLAG tag
Clone 1	QVQLQQSGPE LVKPGASVKLS CKASGYTFS	NYWM Q	WVKQR PGQGL EWIG	AIYPEDGDT RYTQKFKG	KATLTADKSSTTAYMQ LSSLTSEDSAVYYCAR	DGHYGM DY	WGQGT SVTVSS	DYKDDDDK
Clone 2	QVQLQQSGA ELVRPGALVKL SCKASGFNIK	DYYMH	WVKQR PEQGLE WIG	WIDPENGN TIYDPKFQG	KASITADTSSNTAYLQL SSLTSEDTADTSSNTAY LQLSSLTSEDTAVYYCV R	DNYGYDA FGY	WGQGT LVTASS	DYKDDDDK
Clone 3	DVKLQESGAE LVRPGALVKLS CKASDFNIK	DYYMH	WVKQR PEQGLE WIG	WIDPENGN TIYDPKFQG	KASITADTSSNTAYLQL SSLTSEDTAVYYCAR	EDYGYDY VPPFDY	WGQGT TLTVSS	DYKDDDDK
Clone 4	EVMLVESGAE LVKPGASVKLS CTTSGFNIK	DYYMH	WVKQR PEQGLE WIG	WIDPENGN AIYDPKFQG	KASITAETSSNTAYLQLS SLTSEDTAVYYCAR	DNYGYYD GFAY	WGQGT LVTVSA	DYKDDDDK
Clone 5	QVQLQQSGA ELVRPGALVKL SCKASGFNIK	DYYMH	WVKQR PEQGLE WIG	WIDPENGN TIYDPKFQG	KASITADTSSNTAYLQL SSLTSEDTAVYYCAR	DNYGYDA FGY	WGQGT LVTASS	DYKDDDDK
Clone 6	EVQLQQSGAE LVRPGTSVKVS CKASGYAFT	NYLIE	WVKLR PGQGL EWIG	VINPGSGG TNYNEKFK G	KATLTADKSSSTAYMQL SSLTSDDSAVYFCAR	DGVYYRY DEGNYFA MDY	WGQGT SVTVSS	DYKDDDDK

Table Amino acid sequence of hCL1 binders.
The amino acids sequences of scFv clones were analyzed.

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分担研究報告書

scFv 提示ファージライブラリの構築

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研究要旨

現在までに C 型肝炎ウイルスの感染受容体として、claudin(CL)-1 、occludin、CD81、Scavenger receptor class B type I(SR-BI)などが同定され、感染受容体アンタゴニストを利用したC型肝炎ウイルス感染阻害法の可能性が提唱された。2010 年に抗 CL-1 抗体が多様な genotype の C 型肝炎ウイルスに対し感染阻害活性をもつことが示されたことから、CL-1 アンタゴニストが C 型肝炎ウイルス感染阻害薬の候補として注目されている。しかしながら、CL-1 アンタゴニストの創製に成功した例は唯一この報告のみであり、druggable CL-1 binder の開発は未だ皆無である。

一本鎖抗体(scFv)は、抗体の可変領域(VH, VL)のみから構成され、IgG に比べ分子量が約 1/5 と低分子であることから、血管から組織への移行性に優れており、大腸菌による組換え蛋白質として産生可能であることから、製造コストが低く抑えられるなど創薬上のメリットがある。このように、scFv を利用とした CL-1 アンタゴニストは druggable CL-1 binder としての可能性を秘めている、膜蛋白質である CL は精製が困難であり、抗原性も低いためその創製は立ち遅れているのが現状である。

本年度は、hCL1 提示バキュロウイルスで免疫誘導した gp64 トランスジェニックマウスから、2.6×10^5 CFU サイズのファージディスプレイ scFv ライブラリを構築した。

A. 研究目的

現在までに、C 型肝炎ウイルス(HCV)の受容体として、CD81、claudin(CL)-1 、occludin、Scavenger receptor class B type I(SR-BI)などが同定されている。HCV のエンベロープ蛋白質である E2 蛋白質がこれら感染受容体に結合すると、HCV 粒子はエンドサイトーシスにより細胞内に取り込まれる。従って、HCV 感染受容体の細胞外領域に結合する分子は、感染阻害作用を有する C 型肝炎治療薬になり得る。実際、2010 年に抗 CL-1 抗体が多様な genotype の HCV に対し、感染阻害活性をもつことが示され、CL-1 アンタゴニストが C 型肝炎ウイルス感染阻害薬として有望であることが示された。しかしながら、CL-1 アンタゴニストの創製に成功した例は唯一この報告のみであり、druggable CL-1 binder の開発は立ち遅れているのが現状である。

本研究は、HCV感染受容体の1つであるCL-1をターゲットとしたC型肝炎治療および予防薬を、独自のファージディスプレイscFvライブラリ作製技術を利用することにより創製しようとするものであり、我が国の健康増進といった社会的側面のみならず、バイオメーカーの育成や知的財産の確保にも大きく貢献できるものである。

本年度は、gp64トランスジェニックマウスにhCL1提示バキュロウイルスを免疫することにより得たhCL1抗体産生マウスを利用し、scFvライブラリの構築を試みた。

B. 研究方法

scFv ライブラリの作製

B. 1　scFv ファージライブラリの構築

hCL1-BV を免疫した gp64 トランスジェニックマウスから脾臓を摘出し、total RNA を回収した。Total RNA から mRNA を精製し、cDNA を合成した。cDNA 4 μl を鋳型として forward primer set 2 μl、reverse primer set 2 μl、PCR buffer 5 μl、dNTP 5 μl、$MgSO_4$ 2 μl、DMSO 1 μl、KOD-plus 1 μl の割合で混合したものをアニーリング温度 50°C で 30 sec 、伸長反応 68°C で 1 min に設定した 35 サイクルの PCR 反応に供し、それぞれ VH 鎖、VL 鎖の cDNA を得た。この PCR 産物を PCR purification kit (QIAGEN) で精製し、続く PCR による VH, VL の連結、増幅 (assembly PCR) に供した。VH 鎖 cDNA を 100 ng、VL 鎖 cDNA を 100 ng、PCR buffer 5 μl、dNTP 5 μl、$MgSO_4$ 2 μl、DMSO 1 μl、KOD-plus 1 μl の割合で混合したものをアニーリング温度 65°C で 1 分間 、伸長反応 68°C で 1 分間に設定した 18 サイクルの条件に設定し、assembly PCR を行った。さらに scFv 100 ng、Not I サイトを有する Y15 primer (5'-ggccagctttggagccttttttttggagattttcaacgtgaaaaaatt atttattcgcaattcctttagttgttcctttctatgcggcccagccggccat ggcc-3') 0.4 μl、Nco I サイトを有する Y16 primer (5'-ttagtaaatgaattttctgtatgaggttttgctaaacaactttcaacag tctatgcggcacgcggttccacggatccggatacggcaccggcgcacct gcggccgc-3') 0.4 μl、PCR buffer 5 μl、dNTP 5 μl、$MgSO_4$ 2 μl、DMSO 1 μl、KOD-plus 1 μl の割合で混合したものをアニーリング温度 65°C で 1 分間 、伸長反応 68°C で 1 分間に設定した 35 サイクルの条件に設定し PCR を行い、scFv を増幅した。PCR 産物を PCR purification kit を用いて精製し、scFv 遺伝子とした。scFv 遺伝子を Nco I 、Not I で 37°C、20 時間処理し、切り出し精製を行った。同様に Nco I、Not I で 2 h 処理し、切り出し精製した pY03'-importin α を 1 μg、scFv 遺伝子を 0.8 μg 用いて T4 DNA ligase を用いて 16°C にて一晩ライゲーション反応を行なった。得られたライゲーション産物を PCR Purification Kit で精製した。ライゲーション産物を大腸菌 TG1 (STRATAGENE) にエレクトロポレーションすることにより導入した。その後、100 μg/ml ampicillin sodium と終濃度 2% D-glucose を添加した LB 培地 (LAG 培地) プレートに播種した。一晩培養後の大腸菌のコロニーをセルスクレーパーにより LAG 培地で回収した。この大腸菌溶液を終濃度 10%となるようにグリセロール (ナカライテスク) を添加して -80°C で保存し、hCL1-BV 免疫 gp64Tg ライブラリとした。

B. 2　エレクトロポレーション

TG1 をグリセロールストックから 2YT (2-YT BROTH, Invitrogen) 培地 2 ml で一晩培養した。翌日、2YT 培地 200 ml に OD600 : 0.05-0.1 となるように植え継ぎ、37°C で OD600 : 0.4-0.6 まで培養した。その後、4°C、3000 rpm 10 分間遠心分離し、上清を捨て、milliQ 水を加え懸濁し、さらに 4°C、3000 rpm 10 分間遠心分離し、上清を捨てた。この洗浄作業を3回繰り返した後、TG1 を終濃度 10%のグリセロールを含む SP 水で懸濁した。TG1 溶液 50 μl とライゲーション産物 1 μl (30 セット) を氷上で 15 分間なじませた後、混合液をキュベットに移し、Gene pulser® (Bio-Rad Laboratories) を用いて電気パルスを与えた (Ec1)。その後、終濃度 2% D-glucose を添加した 2YT (2YTG) 培地 950 μl に移し、37°C で 1 時間振盪培養した。Titer check 用として、この大腸菌溶液のうち 50 μl を 100 μg/ml ampicillin sodium を添加した 2YTG (2YTGA) 培地で 10^2-10^6 倍希釈し、ペトリフィルム (3M Microbiology Products) に播き、37°C で一晩培養後、コロニー数を計測することでライブラリのサイズを求めた。また、残りの大腸菌溶液をプレート 1 枚あたり約 300 μl となるように LAG 培地プレート 40 枚に播種した。翌日、プレート 1 枚あたり 2 ml の LAG 培地でセルスクレーパーを用いて大腸菌を回収し、終濃度 10%となるようにグリセロールを添加し、-80°C で保存した。

B. 3　scFv ファージライブラリの多様性確認

エレクトロポレーションの際に播種したペトリフィルムからコロニーをランダムにピックアップし、LA 培地 3 ml で一晩培養した。その後、ミニプレップにより plasmid を精製した。精製した plasmid を鋳型として、primer とし

て pY03'-S-1 (5'-caggaaacagctatgac-3') を用い、シークエンス解析を行った。

C. 研究結果

gp64 トランスジェニックマウスに hCL1-BV を免疫したマウスの脾臓から作製した cDNA を鋳型に、重鎖可変領域 (VH) 及び軽鎖可変領域 (VL) の遺伝子を PCR 法により増幅した。VH、VL 遺伝子をリンカーで連結して得た scFv 遺伝子を Nco I/ Not I 処理し、ファージディスプレイ用ベクターである pY03' に組み込んだ。得られた産物をエレクトロポレーション法により大腸菌 TG-1 に導入したものを scFv 提示ファージライブラリとした。構築した scFv ライブラリのライブラリサイズは、2.6×10^5 CFU だった。ライブラリの多様性を確認するため、ランダムに選出したクローンのシークエンス解析を行った (Table)。

D. 考察

免疫ライブラリは、10^5-10^6 CFU 程度のライブラリサイズを有し、非免疫ライブラリと比して多様性の面では劣るが、抗原への指向性の面では優れている。今回構築した scFv 提示ファージライブラリのライブラリサイズは 2.6×10^5 CFU であったことから、十分なライブラリサイズであると考えられる。抗体遺伝子の配列の多様性は抗原との結合に重要な VH 鎖の CDR3 領域において見られることから、作製したライブラリの VH 鎖 CDR3 領域を調べたところ、クローン間でアミノ酸配列に多様性が認められた(Table)。従って、本ライブラリは hCL1 binder 取得に際し有用なスクリーニングソースであると考え、続く hCL1 binder スクリーニング実験に供した。

E. 結論

本研究は、gp64トランスジェニックマウスにhCL1提示バキュロウイルスを免疫することにより得たhCL1抗体産生マウスを利用し、scFv提示ファージライブラリの作製を行った。その結果、hCL1結合性scFv取得に十分なライブラリの構築に成功した。

F. 健康危険情報

該当事項なし

G. 研究発表

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H. 知的財産権の出願・登録状況

1. 特許取得

該当事項なし

2 実用新案登録

該当事項なし

3. その他

該当事項なし

VL	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4	(G4S)3
Clone 1	GIVMTQSPT FLAVTASKK VTISC	TASESLY SSKHKVH YLA	WYQKKPE QSPKLLIY	GASNRYI	GVPDRFTGSGS GTDFTLTISSVQV EDLTHYYC	AQFYSYP LT	FGAGTK LEIKR	GGGGSGG GGSGGGG S
Clone 2	DIVMTQSQK FMSTSVGD RVSVTC	KASQNVG TNVA	WYQQKPG QSPKALIY	SASYRY S	GVPDRFTGSGS GTDFTLTISNVQS EDLADYFC	QQYSNYY T	FGGGT KLELER	GGGGSGG GSGGGGS
Clone 3	DIVMTQSHK FMSTSVGE RVNITC	KASQDVS TAVA	WYQQKPG QSPKLLIY	SASYRY T	GVPDRFTGSGS GTDFTFTISSVQA EDLAVYYC	QQYNSYP LT	FGAGTK LELKR	GGGGSGG GGSGGGG S
Clone 4	DIVMTQSHK IMSTSVGDG VSITC	KASQDVS PAVA	WYQQKPG QSPKLLIY	SASYRY S	GVPDRFTGSGS GTDFTLTISNVQS EDLAEYFC	QQYNSYP YT	FGGGT KLELKR	GGGGSGG GGSGGGG S
Clone 5	DIQMTQSH KFMSTSVG DRVSITC	KASPDVS TAVA	WYQQKPG QSPQLLIY	SASYRY T	GVPDRFTGSGS GTDFTFTISSVQA EDLAVYYC	QQHYSTP LT	FGAGTK LEIKR	GGGGSGG GGSGGGG S
Clone 6	DIVMTQSQK FMSTSVGD RVSITC	KASQDVG TAVA	WYQQKPG QSPSKLLIY	WASTRH T	GVPDRFTGSGS GTDFTLTISNVQS EDLAEYFC	QQYNTYP LT	FGAGTK LEIKR	GGGGSGG GGSGGGG S
Clone 7	DIVMTQSHK FMSTSVRD RVSITC	KASQNVG TNVA	WYQQKPG QSPKALIY	SASYRY S	GVPDRFTGSGS GTDFTLTISNVQS EDLAEYFC	QQYNYYP LT	FGAGTK LEIKR	GGGGSGG GGSGGGG S
Clone 8	DIVITQSHKF MSTSVGDR VSITC	KASQDVS TAVA	WYQQKPG QSPKLLIY	SASYRY T	GVPDRFTGSGS GTDFTFTISSVQA EDLAVYYC	QQHYSTP YT	FGGGT KLELKR	GGGGSGG GGSGGGG S

VH	FR1	CDR 1	FR2	CDR2	FR3	CDR3	FR4	FLAG
Clone 1	DVKLVESGGG LVKPGGSLKLS CAASGFTFS	SYTMS	WVRQT PEKRL EWVA	TISSGGGY TYYLDTVK G	RFTISRDNAKNNLY LQMSSLRSEDTALY YCAR	RSLDGY DYWYFD V	WGAGT TLTVSS	DYKDDDDK
Clone 2	EVKLVESGGDL VKPGGSLKLS CAASGFTFS	SYGMS	WVRQT PDKRL EWVA	TISSGGSF TYYPDSVK G	RFTISRDNAKNTLH LQMSSLKSEDTAM YYCAR	HGSSYY AMDY	WGQGT SVTVSS	DYKDDDDK
Clone 3	EVQLQQSGDD LVKPGASVKLS CKASGYTFT	SYWIN	WIKQR PGQGL EWIG	RIAPGSGS TYYNEMFK G	KATLTVDTSSSTAYI QLSSLSSEDSAVYF FCAR	RGIWGS SYDYFD Y	WGQGT TLTVSS	DYKDDDDK
Clone 4	QVQLKQSGAE LVRPGALVKLS CKASGFNIK	DYFMH	WVKQ RPEQG LEWIG	WIDPENGN TIYDPKFQ G	KASITADTSSNTAYL QLSSLTSEDTAVYY CAR	RYRWYL SHFDY	WGQGT TLTVSS	DYKDDDDK
Clone 5	EVMLVESGGG LVQPGGSRKL SCAASGFTFS	NYAMS	WGRQ TPDKR LEWVA	TITSGGSY TYYPDSVK G	RFTISRANAKHTLY LRMSSLRSEDTAM YYCTR	HEDTLLR RHFDY	WGQGT TLTVSS	DYKDDDDK
Clone 6	EVKLVESGGGL VKPGGSLKLS CAASGFTFS	SYAMS	WVRQT PEKRL EWVA	TISGGGTT YYPDSVKG	RFTISRDNAKNNLY LQMSSLRSEDTALY YCAR	DDYDET GSFAY	WGQGT LVTVSS	DYKDDDDK
Clone 7	EVMLVESGGG LVKPGGSLKLS CAASGFTLS	SYAMS	WVRQ SPEKR LEWVA	EISSGGSY TYYPDTVT G	RFTISRDNAKNTLY LEMSSLRSEDTAM YYCAR	VVYYAM DY	WGQGT TLTVSA	DYKDDDDK
Clone 8	EVMLVESGGG LVKPGGSLKLS CAASGFTFS	SYAMS	WVRQT PEKRL EWVA	TISSGGSY NYYPDSVK G	RFITSRDNAKNTLY LQMSSLRSEDTAM YYCAR	QGPPFA Y	WGQGT LVSVSS	DYKDDDDK

Table Amino acid sequence of scFv phage library.
Phage clones were randomly picked up from the scFv phage library, and the amino acids sequences of scFv clones were analyzed.

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
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雑誌

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Watari A, Yagi K, Kondoh M.	A simple reporter assay for screening claudin-4 modulators.	Biochem. Biophys. Res. Commun	426(4)	454-60	2012
Yamagishi Y., Watari A., Hayata Y., Li X., Kondoh M., Tsutsumi Y., Yagi K.	Hepatotoxicity of sub-nanosized platinum particles in mice.	Pharmazie,	68(3)	178-82	2013
Kitagaki M., Isoda K., Kamada H., Kobayashi T., Tsunoda S., Tsutsumi Y., Niida T., Kujiraoka T., Ishigami N., Ishihara M., Matsubara O., Ohsuzu F., Kikuchi M.	Novel TNF-alpha receptor-1 antagonist treatment attenuates arterial inflammation and intimal hyperplasia in mice.	J. Atheroscler. Thromb	19(1)	36-46	2012
Yamashita T., Okamura T., Nagano K., Imai S., Abe Y., Nabeshi H., Yoshikawa T., Yoshioka Y., Kamada H., Tsutsumi Y., Tsunoda S.	Rho GDP-dissociation inhibitor alpha is associated with cancer metastasis in colon and prostate cancer.	Pharmazie	67(3)	253-5	2012

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Morishige T., Yoshioka Y., Narimatsu S., Ikemizu S., Tsunoda S., Tsutsumi Y., Mukai Y., Okada N., Nakagawa S.	Mutants of lymphotoxin-α with augmented cytotoxic activity via TNFR1 for use in cancer therapy	Cytokine	61(2)	578-84	2012
Yoshikawa M., Mukai Y., Okada Y., Tsumori Y., Tsunoda S., Tsutsumi Y., Aird WC., Yoshioka Y., Okada N., Doi T., Nakagawa S.	Robo4 is an effective tumor endothelial marker for antibody-drug conjugates based on the rapid isolation of the anti-Robo4 cell-internalizing antibody.	Blood	121(14)	2804-13	2013

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A simple reporter assay for screening claudin-4 modulators

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ABSTRACT

Claudin-4, a member of a tetra-transmembrane protein family that comprises 27 members, is a key functional and structural component of the tight junction-seal in mucosal epithelium. Modulation of the claudin-4-barrier for drug absorption is now of research interest. Disruption of the claudin-4-seal occurs during inflammation. Therefore, claudin-4 modulators (repressors and inducers) are promising candidates for drug development. However, claudin-4 modulators have never been fully developed. Here, we attempted to design a screening system for claudin-4 modulators by using a reporter assay. We prepared a plasmid vector coding a claudin-4 promoter-driven luciferase gene and established stable reporter gene-expressing cells. We identified thiabendazole, carotene and curcumin as claudin-4 inducers, and potassium carbonate as a claudin-4 repressor by using the reporter cells. They also increased or decreased, respectively, the integrity of the tight junction-seal in Caco-2 cells. This simple reporter system will be a powerful tool for the development of claudin-4 modulators.

1. Introduction

Tight junctions (TJs), the most apical components of intercellular junctional complexes, function as fences that maintain cellular polarity and provide a barrier to regulate intercellular permeability of epithelia [1,2]. Disruption of cellular polarity and the TJ-seal is frequently observed during carcinogenesis and inflammation [3]. Modulation of TJ-seals for drug absorption is now of research interest [4,5]. A series of studies has revealed that TJs are composed of transmembrane proteins (such as occludin and claudins), junction adhesion proteins, and cytoplasmic scaffolding proteins, including ZO-1, ZO-2, and ZO-3 (see reviews [6–8]). Of these, claudins are thought to be the main structural and functional components of TJs.

Claudins, tetra-transmembrane proteins with a molecular mass of approximately 23 kDa, comprise a multigene family containing over 20 members [8]. The barrier-function and the expression patterns of claudin members differ among tissues [6,8,9]. Claudin-1-, -5-, and -11-deficient mice show dysfunction of the epidermal barrier, blood–brain barrier, and blood–testis barrier, respectively [10–12]. The expression levels and the barrier-functions of claudins are often altered in various cancer cells; they can be down-regulated or up-regulated, depending on the type of cancer [13]. Changes in claudin expression have also been observed in the mucosal epithelium under inflammatory conditions [14]. Claudins are thus potent targets for drug development, such as drug delivery, anti-cancer agents, and anti-inflammatory agents.

Since claudins play a role in TJ-seals, modulation of the claudin-barrier is a potent strategy for drug absorption. The carboxyl-terminus of *Clostridium perfringens* enterotoxin (C-CPE) is a modulator of the claudin-barrier [15]. Treatment of cells with C-CPE causes a decrease in claudin-4 proteins in TJs, followed by an enhancement of the paracellular transport of solutes without causing cytotoxicity [15]. C-CPE also enhances jejunal, nasal, and pulmonary absorption of drugs [16]. Thus, proof-of-concept for claudin-targeted drug absorption has been demonstrated. A decrease in claudin-4 in the intestinal epithelium often occurs in colitis [17]. Down-regulation of claudin-4 is also observed in some cancer cells [18]. Induction of claudin-4 is involved in the chemo-preventive effect of nonsteroidal anti-inflammatory drugs [19]. A modulator of claudin-4 expression would therefore be a potent molecule for claudin-targeted drug absorption and drug development for some inflammatory diseases and cancers. However, an effective system to screen for claudin modulators is lacking.

Here, we developed a simple system to monitor claudin-4 expression using a reporter gene, and we screened chemical claudin-4 modulators.

Abbreviations: TJs, tight junctions; C-CPE, the carboxyl terminus of *Clostridium perfringens* enterotoxin; TGF-β, transforming growth factor-β; EGF, epidermal growth factor; PMA, phorbol 12-myristate 13-acetate; DMSO, dimethyl sulfoxide; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; qPCR, quantitative PCR; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TER, transepithelial electric resistance.

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2. Materials and methods

2.1. Reagents and cells

Recombinant human transforming growth factor-β (TGF-β) and epidermal growth factor (EGF) were purchased from R&D systems (Minneapolis, MN) and Peprotech Inc. (Rocky Hill, NJ), respectively. The recombinant proteins were dissolved in water and stored at −80 °C before use. Phorbol 12-myristate 13-acetate (PMA) were dissolved in dimethyl sulfoxide (DMSO) and stored at −20 °C before use. List of the chemicals used in this study for screening for claudin-4 modulator is shown in Table 1. All reagents were of research grade.

MCF-7, and Caco-2 cells were cultured in Dulbecco's modified minimal essential medium supplemented with 10% fetal bovine serum in 5% CO_2 at 37 °C. MCF-7 cells were obtained from the RIKEN cell bank (Ibaragi, Japan). Caco-2 cells were obtained from the American Type Culture Collection (Manassas, VA). MCF-7 cells stably expressing snail or HRasV12 were prepared by infection with a recombinant retroviral vector coding for snail or HRasV12 gene.

2.2. Preparation of a reporter plasmid

Genomic DNA was extracted from MCF-7 cells by using a genomic DNA isolation kit (Sigma–Aldrich, St. Louis, MO). The claudin-4 promoter region was cloned by polymerase chain reaction (PCR) using genomic DNA as a template and paired primers (forward primer, 5′-GCGCTAGCGGTTGCCCCCTGGCCTTAAC-3′; reverse primer, 5′-CGCTCGAGGTCCACGGGAGTTGAGGACC-3′). The resultant fragments (500 bp) were subcloned into the pGV-B2 vector encoding the luciferase gene (Toyobo, Osaka, Japan). The sequence of the claudin-4 promoter region was confirmed.

2.3. A transient expression of transfection snail or HRasV12 gene

Transfection was performed with FuGENE HD (Roche, Mannheim, Germany) according to the manufacturer's protocol. Briefly, cells were seeded onto 24-well plates. When the cells reached to 80% confluent cell density, 20 μl of medium containing 0.6 μl of FuGENE HD and 200 ng of plasmid carrying snail or HRasV12 gene was added to the wells. After 48 h of transfection, the luciferase activity of the cell lysates was measured as described below.

2.4. Luciferase assay

Luciferase activity was measured using a commercial available luciferase assay system (Toyo Ink, Tokyo, Japan). Cells were lysed with a cell lysis reagent, LCβ (Toyo Ink). The cell lysates were then centrifuged at *18,000g* for 5 min. The luciferase activity in the resulting supernatant was measured using a TriStar LB 941 microplate reader (Berthold, Wildbad, Germany).

2.5. Establishment of a stable reporter cell line

MCF-7 cells were transfected with the reporter plasmid and a plasmid carrying the puromycin resistance gene. Stable transfectants were selected in the presence of puromycin.

2.6. Screening for claudin-4 modulators

The clone 35 cells were seeded onto 96-well plates at a density of 4×10^4 cells/well. On the following day, vehicle or compound was added, and the cells were cultured for an additional 24 h. The luciferase activity in the cells was then measured as described above.

2.7. Cytotoxicity assay

Clone 35 cells or Caco-2 cells were seeded onto a 96-well plate at a density of 4×10^4 or 6×10^4 cells/well, respectively. On the following day, cells were treated with chemicals at the indicated periods. The cell viability was measured by using a WST-8 assay kit (Nacalai, Kyoto, Japan).

2.8. Reverse transcription–PCR (RT–PCR) analysis

RT reaction and PCR amplification were performed with a cDNA synthesis kit (Roche, Mannheim, Germany) and ExTaqTM (Takara, Shiga, Japan), respectively, according to the manufacturer's instructions. Briefly, total RNA was prepared with TRIzol reagent (Invitrogen, Carlsbad, CA). For reverse transcription, 5 μg of total RNA was used. PCR was performed for 23 cycles for claudin-4 (94 °C for 30 s, 55 °C for 15 s, 72 °C for 30 s) and for 20 cycles for GADPH (94 °C for 30 s, 55 °C for 15 s, 72 °C for 60 s). The PCR products were separated by use of agarose gel electrophoresis and stained with ethidium bromide. The sequences of the primers are as follows: forward primer for claudin-4, 5′-CAACATTGTCACCTCGCAGACCATC-3′; reverse primer for claudin-4, 5′-TATCACCATAAGGCCGGCCAACAG-3′; forward primer for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5′-TCTTCACCACCATGGAGAAG-3′; reverse primer for GAPDH, 5′-ACCACCTGGTGCTCAGTGTA-3′.

2.9. Quantitative PCR (qPCR) analysis

qPCR was performed with SYBR Premix Ex Taq II (Takara) using an Applied Biosystems StepOne Plus (Applied Biosystems, Foster City, CA). Relative quantification was performed against a standard curve and the values were normalized against the input determined for the housekeeping gene, GAPDH. The primer sequences used for qPCR were as follows: forward primer for claudin-4, 5′-TTGTCACCTCGCAGACCATC-3′ and reverse primer for claudin-4, 5′-CAGCGAGTCGTACACCTTG-3′; forward primer for GAPDH, 5′-GGTGGTCTCCTCTGACTTCAACA-3′ and reverse primer for GAPDH, 5′-GTGGTCGTTGAGGGCAATG-3′.

2.10. Western blot analysis

Cells were lysed with RIPA buffer (0.15 M NaCl, 50 mM Tris–HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% protease inhibitor cocktail [Sigma–Aldrich]). The cell lysates were subjected to 15% SDS–polyacrylamide gel electrophoresis (SDS–PAGE), followed by blotting onto polyvinylidene difluoride membrane. The membranes were incubated with anti-claudin-4 mouse monoclonal (Zymed, South San Francisco, CA) and anti-β-actin mouse monoclonal (Sigma–Aldrich) antibodies, respectively, and subsequently treated with horseradish peroxidase-conjugated anti-mouse IgG (Zymed). The reactive bands were detected by using an enhanced chemiluminescence reagent (GE Healthcare, Buckinghamshire, UK).

2.11. Transepithelial electric resistance (TER) assay

Caco-2 cells were seeded into Transwell™ chambers (Corning, NY) at a density of 8×10^4 cells/well. On 7 days after the seeding or when TER values reached a plateau, claudin-4 inducers (thiabendazole, carotene, or curcumin) or claudin-4 repressor (potassium carbonate), respectively, was added. The TER values were then monitored at 0, 24, and 48 h using a Millicell-ERS epithelial

Table 1
Chemicals used in this study as screening sources.

Sample number	Sample name	Concentration[a]	Relative luciferase activity[b]
1	Tartrazine	10 mM	1.29
2	Potassium nitrate	1 mM	0.94
3	Potassium carbonate	10 mM	0.56
4	Sodium chlorous	10 mM	0.95
5	Zinc sulfate	0.1 mM	0.95
6	New coccine	0.01 mM	0.98
7	Amaranth (Bordeaux S)	1 mM	1.34
8	Allura red AC	1 mM	1.49
9	Sunset yellow FCF	1 mM	1.59
10	Potassium hydroxide	1 mM	0.83
11	L-ascorbic acid	1 mM	1.02
12	Sodium nitrite	10 mM	0.91
13	Propionic acid	0.0001%	0.82
14	Sodium carbonate	1 mM	0.91
15	Zinc gluconate	0.01%	1.76
16	Benzoic acid	0.01 mM	1.3
17	Sorbic acid	1 mM	1.51
18	Aspartame	1 mM	1.59
19	Dibutylhydroxytoluene	0.01 mM	1.81
20	Allyl isothiocyanate	0.0001%	1.72
21	Saccharin	1 mM	1.5
22	L-Ascorbyl palmitate	1 mM	1.21
23	Hydroxy biphenyl	0.01 mM	1.87
24	Aluminium potassium sulfate	0.1 mM	0.94
25	L-Lysine	10 mM	1.42
26	Calcium pantothenate	10 mM	1.61
27	Carrageenin	0.01 mM	1.56
28	Tartaric acid	1 mM	1.01
29	Sodium acetate	10 mM	1.02
30	Glycine	10 mM	1.68
31	Sodium alginate	10 mM	1.52
32	Ammonium chloride	10 mM	1.91
33	Magnesium sulfate	10 mM	1.56
34	5-Ribonucleotide	0.001 mM	1.15
35	Calcium chloride	1 mM	1.62
36	Valine	10 mM	1.08
37	Erythrosine	0.01 mM	1.22
38	Annatto	0.01 mM	1.96
39	Maltitol	10 mM	1.44
40	Sodium dehydroacetate	1 mM	1.98
41	Nicotinic acid	1 mM	1.55
42	Isoleucine	1 mM	1.06
43	Mannitol	10 mM	1.29
44	Ascorbic acid (Vitamin C)	10 mM	1.17
45	Phenylalanine	1 mM	0.95
46	Gallic acid	0.1 mM	1.41
47	Erythorbic acid (Sodium isoascorbate)	1 mM	1.03
48	Magnesium chloride	0.1%	1.26
49	Cochineal extract	0.1%	1.02
50	Calcium dihydrogen pyrophosphate	1 mM	1.1
51	Calcium citrate	0.01 mM	0.92
52	Polyvinyl acetate	0.1 mM	1.13
53	Fumaric acid	0.01 mM	1.24
54	Sodium methyl *p*-hydroxybenzoate	1 mM	2.04
55	Tocophenol (Vitamin E)	0.0001%	2.14
56	Rennet	0.01%	0.89
57	Ionone	0.01%	1.15
58	Isoeugenol	0.001%	1.15
59	Allyl isosulfocyanate	0.001%	1.06
60	Propylene glycol	0.1%	0.87
61	Ethyl isovalerate	0.001%	0.89
62	Pectin	0.001%	0.98
63	Cysteine	0.01 mM	0.76
64	Tragacanth gum	0.01%	0.83
65	Thiamin	0.1%	1.15
66	Gum arabic	0.01%	0.91
67	Cellulose	0.001%	0.84
68	Thiabendazole	0.1 mM	3.24
69	Isopropyl citrate	10 mM	1.04
70	γ-oryzanol	0.01%	1.02
71	Calcium carbonate	0.001%	0.857
72	Propylene glycol alginate	0.01%	0.87
73	Chlorophyll	0.1%	1.02
74	Sodium chondroitin sulfate	0.1%	1.04

Table 1 (*continued*)

Sample number	Sample name	Concentration[a]	Relative luciferase activity[b]
75	Biphenyl	0.1 mM	0.99
76	Sodium cytidylic acid	1 mM	0.77
77	Stevia rebaudiana	0.01%	0.96
78	Calcium stearoyl lactylate	0.01%	0.83
79	Ferrous sulfate	0.1 mM	1.37
80	Calcium sulfate	0.1 mM	0.93
81	Benzoyl peroxide	0.1 mM	1.13
82	Dibenzoyl thiamine	1 mM	0.88
83	Carotene	0.1 mM	2.09
84	Guar gum	0.001%	0.84
85	Xanthan gum	0.001%	0.77
86	Curcumin	0.01 mM	2.0

[a] The chemical concentrations were set at the maximum level to show no cytotoxicity.
[b] The relative luciferase activities were calculated as the ratio of that in the chemical-treated cells to that in the vehicle-treated cells. The treatment period was 24 h.

volt-ohmmeter (Millipore Corporation, Billerica, MA). The TER values were normalized to the area of the Caco-2 cell monolayers, and the TER value of a blank chamber was subtracted.

3. Results

3.1. Preparation of a reporter plasmid encoding a claudin-4-promoter-driven luciferase gene

As a first step toward developing a simple screening system for claudin-4 modulators, we cloned the promoter region of claudin-4. We searched for a region that was highly conserved among animals by using a UCSC Genome Bioinformatics program and cloned a 500 bp fragment corresponding to −293 to +194 bp of the claudin-4 gene. This 500 bp fragment contained various transcription factor-binding sites: an E-box (−276 to −271, −262 to −257, −221 to −216, −19 to −14, +10 to +14), a smad-binding element (SBE; −212 to −209, −103 to −100, −38 to −35), and Sp1 (−66 to −57, −53 to −44) [20,21], indicating that this region is a potent candidate for a regulatory region of claudin-4 expression. We constructed a reporter expression vector, in which the 500 bp fragment was inserted upstream of a luciferase gene Suppl. Fig. 1A). To

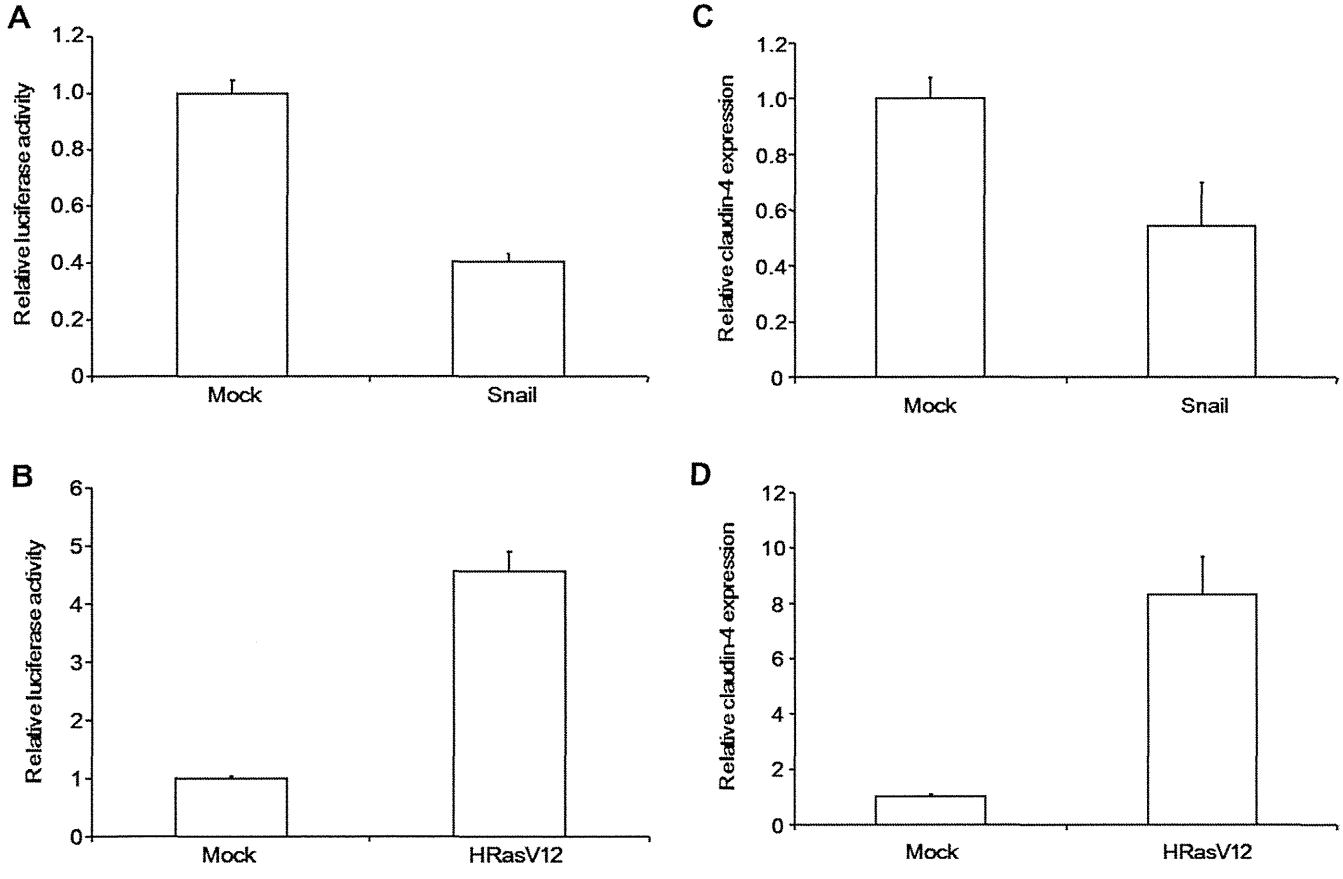

Fig. 1. Preparation of a reporter system monitoring claudin-4 expression. (A, B) Effects of snail and HRasV12 on the luciferase activity in transiently expressing cells. Snail-expressing MCF-7 cells (A) or HRasV12-expressing MCF7 cells (B) were transfected with the claudin-4 reporter plasmid. Two days later, the cells were recovered, and the luciferase activity in the lysates was measured. The data are means ± S.D. ($n = 3$). The results are representative of two independent experiments. (C, D) qPCR analysis of claudin-4 expression in transiently expressing cells. After 2 days of the transfection with the claudin-4 reporter plasmid, total RNA was extracted from snail-expressing MCF-7 cells (C) or HRasV12-expressing MCF-7 cells (D). Expression level of claudin-4 of the transfected cells was quantified by qPCR as described in the Section 2. Claudin-4 expression level was shown as ratio to that of the mock cells. The data are means ± S.D. ($n = 3$). The results are representative of two independent experiments.

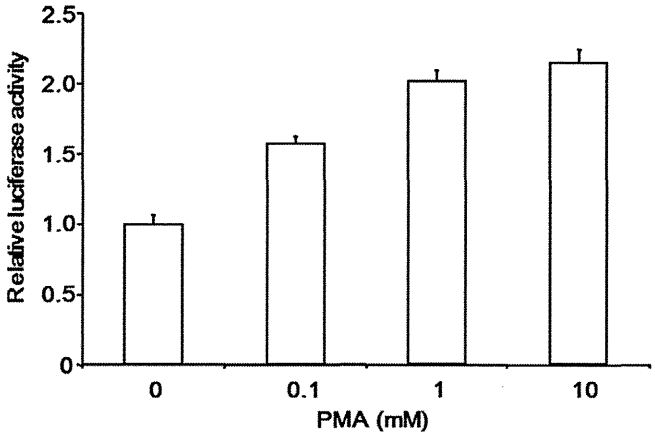

Fig. 2. Effect of PMA on the luciferase activity in clone 35 cells. Clone 35 cells were treated with PMA at the indicated concentrations for 24 h. Luciferase activity in the lysates was measured. The relative luciferase activity is shown as the ratio of the luciferase activity in the treated cells to that of the vehicle-treated cells. The data are means ± S.D. ($n = 3$). The results are representative of two independent experiments.

evaluate expression of the reporter gene, we checked the endogenous claudin-4 expression level in various cell lines and selected MCF-7, HaCat, HT1080, and SiHa cells, which have different claudin-4 expression levels for our analyses (Suppl. Fig. 1B). We transiently transfected the reporter plasmid into these cell lines and found that the luciferase activity of each was correlated with the endogenous expression level of claudin-4 (Suppl. Fig. 1C). We also investigated expression of the reporter gene in MCF-7 cells stably expressing snail or HRasV12, which suppress or induce claudin-4 expression, respectively [22,23]. Transfection of snail- or HRasV12-expressing MCF-7 cells with the reporter plasmid decreased or increased, respectively, the luciferase activity compared to that of mock-transfected MCF-7 cells (Fig. 1A and B). The difference in luciferase activity paralleled the level of claudin-4 mRNA in the cells (Fig. 1C and D), suggesting that the cloned promoter region was functional.

3.2. Preparation of a screening system for claudin-4 modulators

We transfected MCF-7 cells with the claudin-4 reporter plasmid and isolated stable transfected clones. We investigated the effect of transient expression of snail and HRasV12 on luciferase activity in these clones and found that several clones showed altered luciferase activity when transfected with the claudin-4 suppressor (snail, Suppl. Fig. 2A) or the claudin-4 inducer (HRasV12, Suppl. Fig. 2B). TGF-β suppresses claudin-4 expression [23], whereas EGF enhances claudin-4 expression [24]. Therefore, we also investigated the effects of TGF-β and EGF on the luciferase activity in the clones (Suppl. Fig. 2C and D, respectively). Since clone 35 showed the best

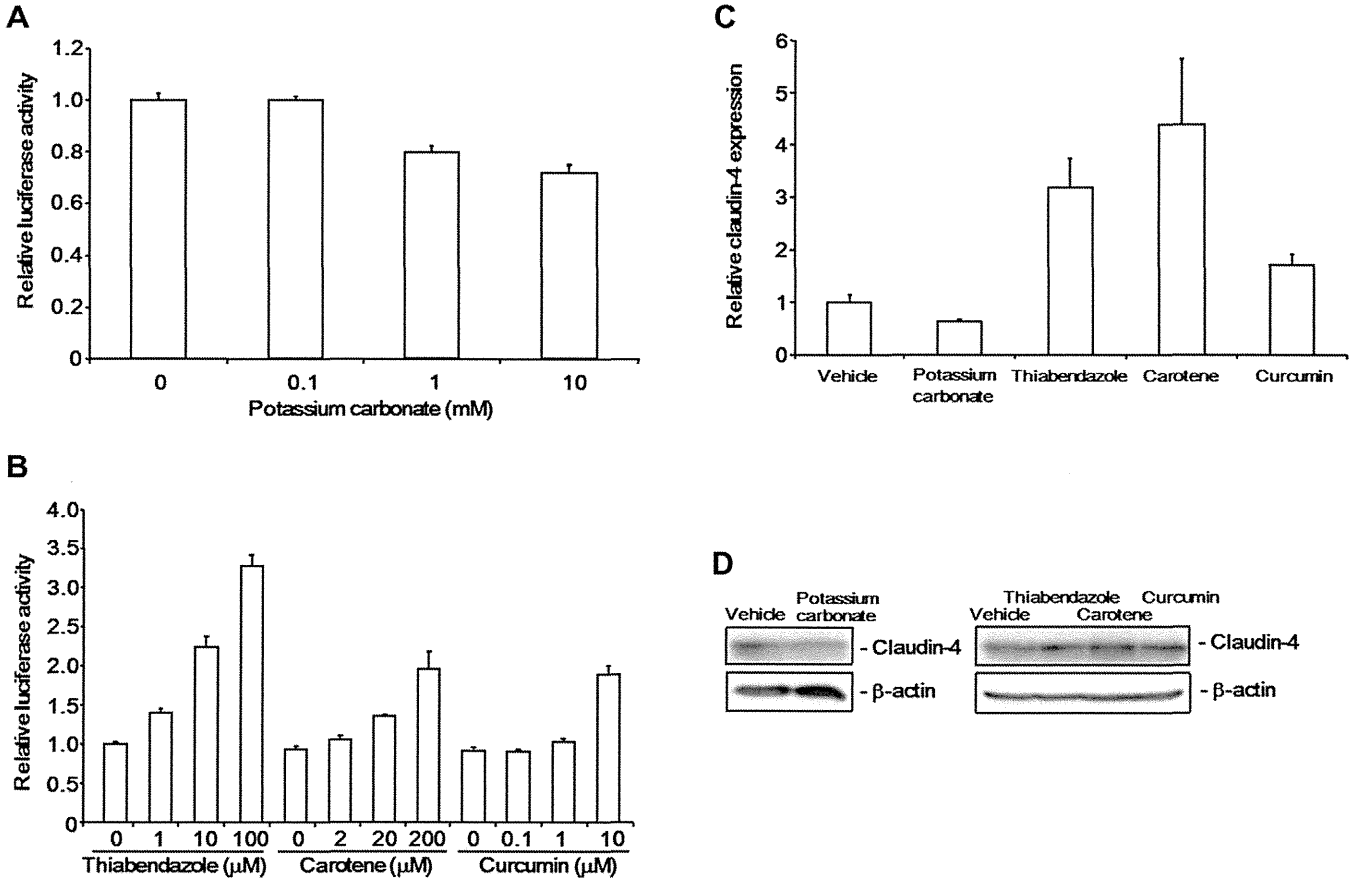

Fig. 3. Screening claudin-4 modulators using the reporter system. (A, B) Dose-dependent effects of the claudin-4 modulator candidates on luciferase expression. Clone 35 cells were treated with potassium carbonate (A), or thiabendazole, carotene, or curcumin (B) at the indicated concentrations for 24 h. Luciferase activity was measured in the lysates. Relative luciferase activity is shown as the ratio of the luciferase activity in the chemical-treated cells to that in the vehicle-treated cells. The data are means ± S.D. ($n = 3$). The results are representative of three independent experiments. (C, D) Effects of the claudin-4 modulator candidates on claudin-4 mRNA expression (C) and claudin-4 protein (D) levels. Clone 35 cells were treated with potassium carbonate (5 mM), thiabendazole (0.1 mM), carotene (0.2 mM), or curcumin (10 μM) for 24 h (C) or 48 h (D). Total RNA was used for qPCR analysis to detect claudin-4 mRNA (C). The relative mRNA expression of claudin-4 normalized to GAPDH expression. The cell lysates were subjected to SDS–PAGE, followed by immunoblotting for claudin-4 (D). GAPDH or β-actin served as loading controls. The result is representative of three independent experiments.

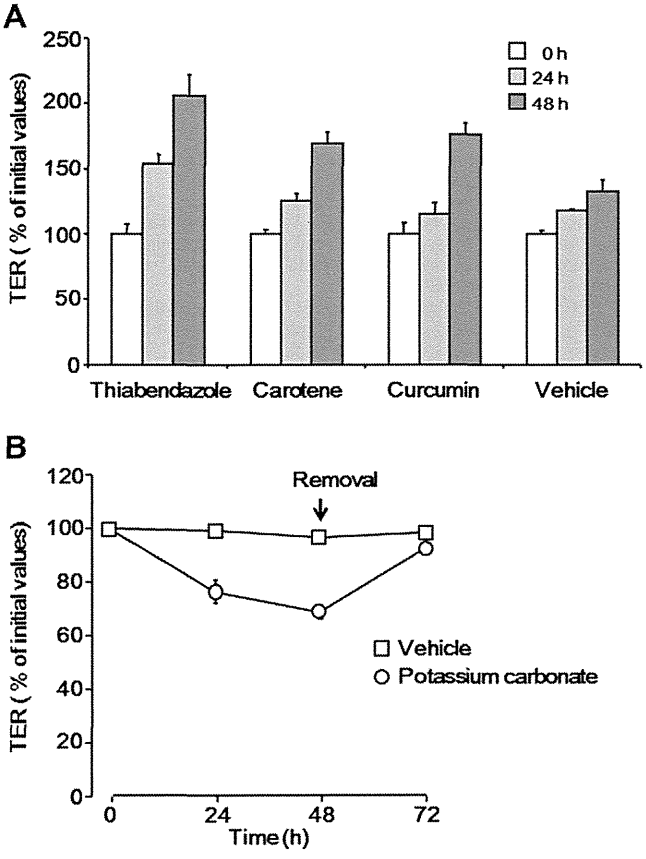

Fig. 4. Effects of claudin-4 modulator on the TJ-barrier in Caco-2 cells. (A) Effect of claudin-4 inducers on the TJ-barrier. Cells were seeded in Transwell™ chambers. Seven days after seeding, the cells were treated with thiabendazole (0.05 mM), carotene (0.2 mM), or curcumin (10 μM). TER values were monitored every 24 h. (B) Effect of a claudin-4 repressor on the TJ-barrier. Cells were seeded in Transwell™ chambers. When the TER values reached a plateau, the TJ-developed cells were treated with potassium carbonate (10 mM). After 48 h of treatment, the medium was replaced with fresh medium. The cells were then cultured for an additional 24 h. TER values were monitored every 24 h. TER values are shown as percentages of the TER values before treatment relative to those in treated cells, as described in the Section 2. The data are means ± S.D. ($n = 3$). These results are representative of three independent experiments.

response to the various claudin-4-modulating treatments, we selected it for further analysis. The clone 35 cells were treated with PMA, which enhances claudin-4 expression [25]. PMA increased luciferase activity in a dose-dependent manner (Fig. 2). These results indicate that clone 35 could be used to screen for modulators of claudin-4 expression.

3.3. Screening for claudin-4 modulators

When we eat, fragments of partially digested food, which still have antigenicity, exist in the intestine. This suggests that claudin modulators that tighten TJ-barriers may be contained in food. Therefore, we screened 86 chemicals used as food additives for claudin-4 modulators (Table 1). At first, we checked the cytotoxicity of these compounds in the clone 35 cells (Table 1). Then, we treated the cells with the compounds at non-toxic concentrations and identified the following claudin-4 modulator candidates: potassium carbonate (No. 3), thiabendazole (No. 68), carotene (No. 83), and curcumin (No. 86) (Suppl. Fig. 3). Each chemical modulated luciferase activity in a dose-dependent manner (Fig. 3A and B). qPCR analysis revealed that thiabendazole, carotene, and curcumin increased claudin-4 expression in the clone 35 cells (Fig. 3C), whereas potassium carbonate decreased claudin-4 expression. Similar results were obtained from Western blot analysis of claudin-4 (Fig. 3D).

To test whether the screened compounds also modulated the TJ-barrier, we investigated the effect of the compounds on the TER value, a marker of TJ-integrity, in Caco-2 cell monolayers, which is a popular model for mucosal barrier. Treatment of cells with thiabendazole, carotene, and curcumin increased the TER values (Fig. 4A). In contrast, potassium carbonate decreased the TER value. Moreover, the TER values recovered when the potassium carbonate was removed (Fig. 4B), and treatment with potassium carbonate did not cause cytotoxicity (data not shown). Thus, we successfully identified claudin-4 modulators.

4. Discussion

Claudin-4 inducers have been the focus of attention in drug development to treat inflammatory diseases and cancers [17–19]; however, their development has been slow. Some chemicals that modulate TJ integrity have been identified: glutamine, bryostatin-1, berberine, quercetin, and butyrate [26–30]. Here, we established a simple monitoring system for claudin-4 expression using a reporter gene, luciferase, and successfully identified chemical claudin-4 modulators: one suppressor of claudin-4 expression, potassium carbonate, and three inducers of claudin-4, thiabendazole, carotene, and curcumin.

Curcumin is an active ingredient of the spice turmeric, which is used in curry powders and as a food preservative. It is also used in traditional medicine to treat various inflammatory conditions, such as arthritis, colitis, and hepatitis [31]. Curcumin has various biological activities, such as anti-inflammatory, anti-oxidant, and anti-cancer effects [32]; however, the underlying mechanisms have never been fully understood. Here, we found that curcumin induces claudin-4 expression and increases TJ integrity. This enhancement of TJ integrity by curcumin may be associated with its therapeutic activities.

Carotene is a precursor of vitamin A. Retinoic acid, a metabolite of vitamin A, enhances TJ integrity in epithelial cells accompanied by expression of claudin-1, -4, and occludin [33]. These findings suggest that metabolized β-carotene-activated expression of claudins enhances the epithelial barrier in Caco-2 cells. Retinoic acid is a biologically active regulator of cell differentiation, proliferation, and apoptosis in various cell types [34]. The activities of retinoic acid are mediated by two types of nuclear receptors: retinoic acid receptors and their heterodimeric counterparts, retinoid X receptors [35]. Specific heterodimer-mediated transcriptional activation increases TJ integrity [36]. The increase in claudin-4 expression and TJ integrity induced by carotene may be caused by the formation of the heterodimer, followed by transcriptional activation.

Thiabendazole is used as a broad spectrum anthelmintic in various animal species and is also used to control parasitic infections in humans [37]. It is also used as an anti-fungal agent for the treatment of fruits [38]. Here, we found that thiabendazole increases claudin-4 expression and TJ integrity, but the mechanism for these activities remains unclear.

Our screening system identified a repressor of intestinal epithelial barrier function as well as three enhancers. We showed that potassium carbonate reduces claudin-4 expression and epithelial barrier function in Caco-2 cells without causing cytotoxicity. Potassium carbonate is used as an acidity regulator, and paracellular permeability is sensitive to pH [39]. Thus, potassium carbonate might reduce epithelial barrier integrity by changing the pH.

In conclusion, we developed the simple screening system for claudin-4 modulator, and we identified several claudin-4 modulators, including three inducers and one repressor. The screening system will thus be a tool for the development of claudin-4

modulators, thereby contributing to basic and pharmaceutical researches.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.08.083.

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Hepatotoxicity of sub-nanosized platinum particles in mice

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Nano-sized materials are widely used in consumer products, medical devices and engineered pharmaceuticals. Advances in nanotechnology have resulted in materials smaller than the nanoscale, but the biologic safety of the sub-nanosized materials has not been fully assessed. In this study, we evaluated the toxic effects of sub-nanosized platinum particles (snPt) in the mouse liver. After intravenous administration of snPt (15 mg/kg body weight) into mice, histological analysis revealed acute hepatic injury, and biochemical analysis showed increased levels of serum markers of liver injury and inflammatory cytokines. In contrast, administration of nano-sized platinum particles did not produce these abnormalities. Furthermore, snPt induced cytotoxicity when directly applied to primary hepatocytes. These data suggest that snPt have the potential to induce hepatotoxicity. These findings provide useful information on the further development of sub-nanosized materials.

1. Introduction

Nanotechnology involves manipulation of matter on the scale of the nanometer and has the potential to improve quality of life via functional products. Nanomaterials are commonly defined as objects with dimensions of 1 to 100 nm and are now widely used in electronics, catalysts, clothing, drugs, diagnostic devices, and cosmetics (Baughman et al. 2002; Patra et al. 2010; Service et al. 2007; Ariga et al. 2010). Recent progress in the field has allowed the creation of sub-nanosized materials that have different physicochemical properties, including improved conductivity, durability and strength. Although these materials may be useful for industrial and scientific purposes, the biologic safety of these materials has not been fully evaluated (Nel et al. 2006; Oberdorster et al. 2005).

Nano-sized platinum particles (nPt) are used for industrial applications and in consumer products, such as cosmetics, supplements and food additives (Gehrke et al. 2011; Horie et al. 2011). The biological influence of exposure to nPt has been previously investigated. For example, nPt has anti-oxidative activity (Watanabe et al. 2009; Onizawa et al. 2009; Kajita et al. 2007), and may be useful for the medical treatment of diseases related to oxidative stress and aging. However, some reports suggest that these substances can induce inflammation in mice or impair DNA integrity (Pelka et al. 2009; Park et al. 2010). Thus, the understanding of the biological influences of nPt has still not been definitively established, and our knowledge regarding the biological effects of sub-nanosized platinum particles (snPt) is severely lacking.

Nano-sized particles can enter and penetrate the lungs, intestines and skin. The degree of penetration depends on the size and surface features of the nano-sized particle. Furthermore, nanoparticles can enter the circulatory system and migrate to various organs, such as the brain, spleen, liver, kidney and muscles (Zhu et al. 2008; Furuyama et al. 2009; Oberdorster et al. 2004; Ai et al. 2011). The liver is a vital organ that is involved in the uptake of nutrients and the elimination of waste products and pathogens from the blood; it is also an important organ for the clearance of nanoparticles. However, some nanoparticles are hepatotoxic (Nishimori et al. 2009a, b; Ji et al. 2009; Cho et al. 2009; Folkmann et al. 2009). In the present study, we investigated the influence of sub-nanosized platinum particles (snPt) on the liver.

2. Investigations and results

To investigate the acute liver toxicity of snPt, we administered snPt (15 mg/kg body weight) into mice by intravenous injection. Histological analysis revealed acute hepatic injury, including vacuole degeneration (Fig. 1). Furthermore, administration of snPt at doses over 15 mg/kg resulted in significant elevation of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels (Fig. 2A and B) and of interleukin-6 (IL-6) levels (Fig. 2C). ALT and AST levels were increased at 3 h to 24 h after intravenous administration at 20 mg/kg snPt (Fig. 3A and B). Cell viability assessment by WST assay demonstrated that direct treatment of isolated hepatocytes with snPt at concentrations of 0.1, 1, 10, 50 and 100 μg/ml resulted in a dose-dependent decrease in hepatocyte viability when compared with vehicle-treated cells (Fig. 4). These observations suggest that snPt induced inflammation and hepatocyte death.

Previous reports showed that biological influences of nanomaterials vary according to material size (Nishimori et al. 2009a, b; Jiang et al. 2008; Oberdorster et al. 2010). Therefore, we examined whether nPt, with a diameter of approximately 15 nm, leads

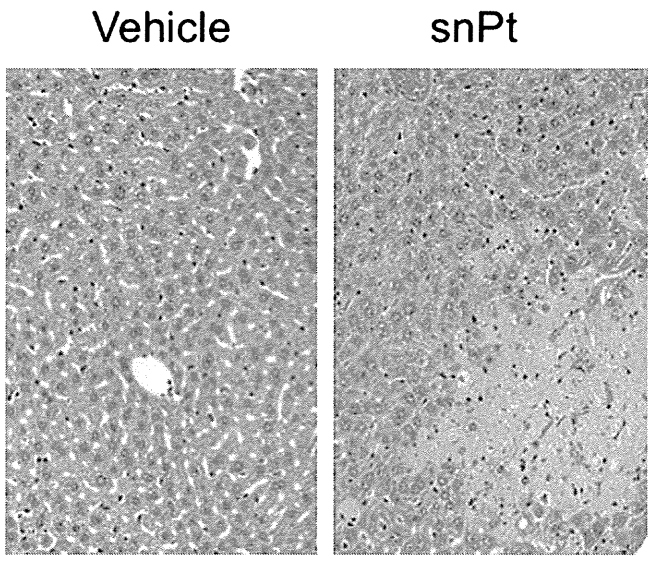

Fig. 1: Histological analysis of liver tissues in snPt-treated mice. snPt was intravenously administered to mice at 15 mg/kg. At 24 h after administration, livers were collected and fixed with 4% paraformaldehyde. Tissue sections were stained with hematoxylin and eosin and observed under a microscope. The pictures show representative data from at least four mice

to a different biologic effect than snPt. As shown in Fig. 5, snPt administration resulted in dose-dependent increases in serum ALT and AST levels, whereas nPt did not. Furthermore, IL-6 levels did not change in response to administration of nPt. These results suggest that the biological effects of platinum particles are dependent on their size.

3. Discussion

The influence of size and of physiochemical properties of nanoparticles on their biologic safety is an important issue. Animal experiments have demonstrated rapid translocation of nanoparticles from the entry site to various organs (Almeida et al. 2011). In particular, nanoparticles tend to concentrate in the liver and are cleared from the body in the feces and urine after intravenous infusion (Ai et al. 2011). While the liver plays a pivotal role in the clearance of nanoparticles, some nanomaterials can induce liver injury. Therefore, we assessed the influence of snPt on the liver and demonstrated that snPt induced liver toxicity *in vitro* and *in vivo*.

Some studies have reported that nPt exert anti-oxidant and anti-inflammatory effects (Watanabe et al. 2009; Onizawa et al. 2009; Kajita et al. 2007), while other studies reported that nPt have negative biological effects. For example, treatment of a human colon carcinoma cell line with nPt resulted in a decrease in cellular glutathione level and impairment in DNA integrity (Pelka et al. 2009). Furthermore, Park et al. (2010) found that nPt prepared from K_2PtCl_6 may induce an inflammatory response in mice. In this study, we found that snPt damaged liver tissues and induced inflammatory cytokines. Kupffer cells present in liver sinusoids may mediate this process via phagocytosis of the particles and subsequent release of inflammatory cytokines. However, when we added snPt to primary hepatocytes, the viability of the cells was significantly reduced, suggesting that snPt may also exert a direct hepatotoxic effect. Thus, the cellular influences of Pt nano- and sub-nano particles may be dependent on the target cells as well as on the size and physical and chemical properties of the particles.

snPt may damage other tissues as well. Cisplatin, a first-line chemotherapy for most cancers, is a platinating agent that can cause kidney damage (Daugaard et al. 1990; Brabec et al. 2005). Furthermore, snPt-induced increases in systemic IL-6 may cause damage to various organs. Further analysis of the distribution and toxic effects of snPt is necessary.

Widespread application of sub-nanosized materials comes with an increased risk of human exposure and environmental release, and the future of nanotechnology will depend on the public acceptance of the risk-benefit ratio. The present study demonstrated that snPt induces hepatotoxicity *in vitro* and *in vivo*. However, our research also indicates that the toxicity of platinum particles could be reduced by altering their size. Additionally, biocompatible coatings can reduce the negative effects of nanoparticles on cells (Oberdorster et al. 2010; Nabeshi et al. 2011; Singh et al. 2007; Clift et al. 2008). Therefore, future studies will contribute to the development of sub-nanosized materials and will also help produce safer products.

4. Experimental

4.1. Materials

Platinum particles with a diameter of 15 nm (nPt) and less than 1 nm (snPt) were purchased from Polytech & Net GmbH (Rostock, Germany). The

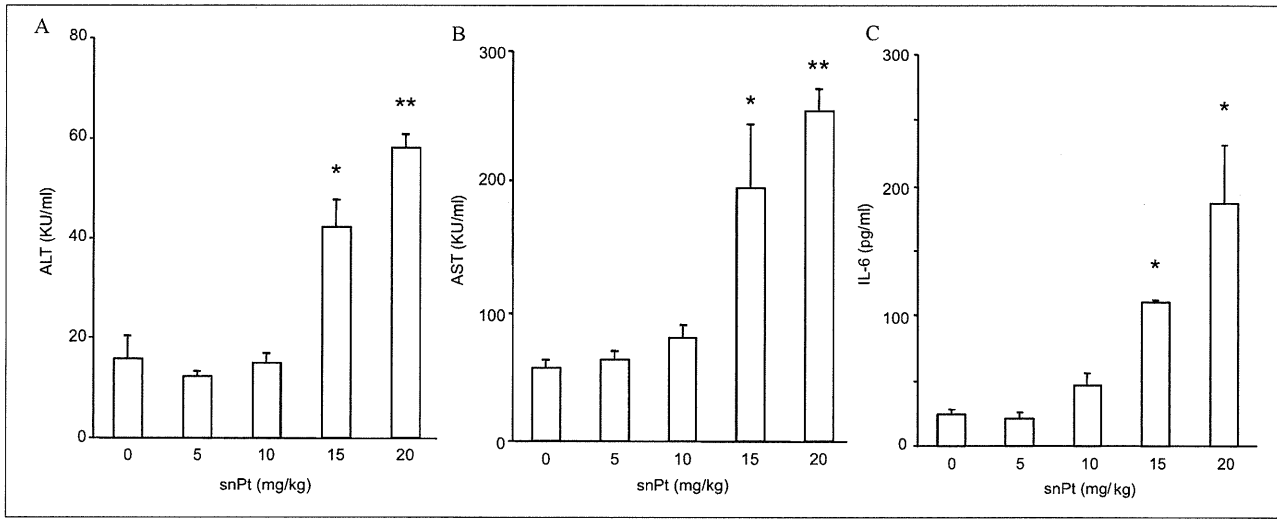

Fig. 2: Dose dependency of snPt-induced liver injury. snPt was intravenously administrated at 5, 10, 15 and 20 mg/kg. At 24 h after administration, blood was recovered, and the resultant serum was used for measurement of ALT (A), AST (B) and IL-6 (C), as described in the "Experimental" section. Data are means ± SEM (n = 3). *Significant difference when compared with the vehicle-treated group (*, $p < 0.05$, **, $p < 0.01$)

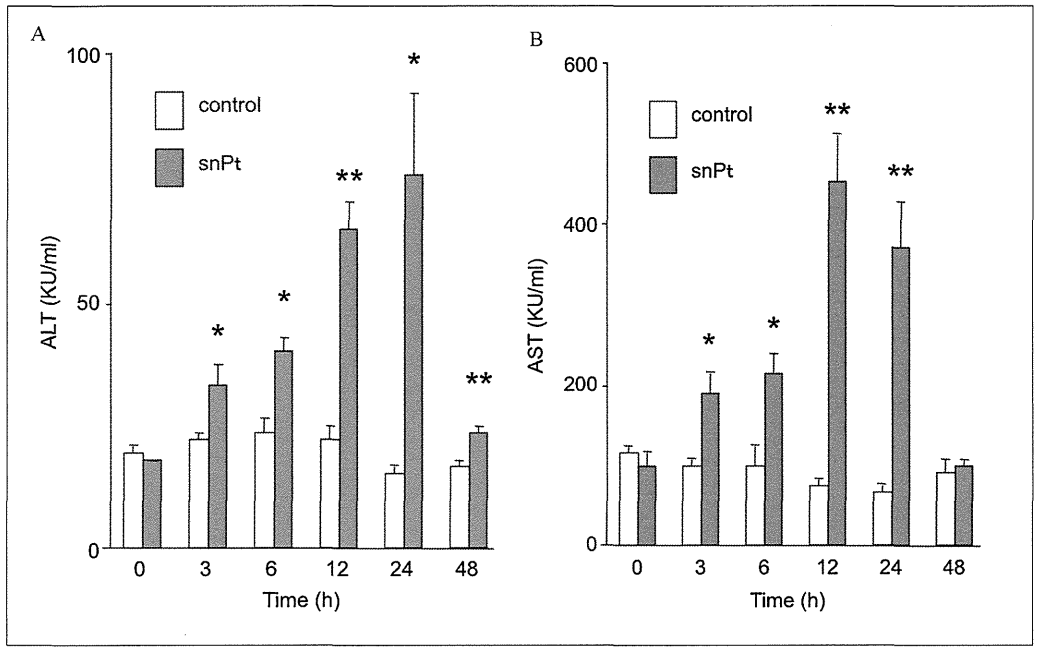

Fig. 3: Time-dependent changes of a biological marker of liver injury. snPt was intravenously administered to mice at 15 mg/kg. Blood was recovered at 3, 6, 12, 24 and 48 h after administration. The serum was used for measurement of ALT (A) and AST (B), as described in the "Experimental" section. Data are means ± SEM (n = 3). *Significant difference when compared with the vehicle-treated group (*, $p < 0.05$, **, $p < 0.01$)

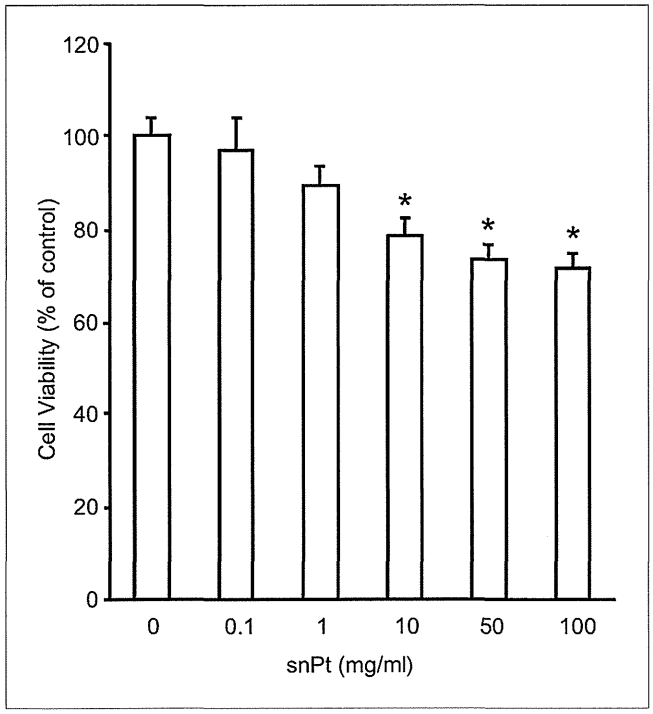

Fig. 4: Cytotoxicity of snPt in hepatic cells. Primary hepatocytes were treated with snPt at 0.1, 1, 10, 50 or 100 µg/ml. After 24 h of culture, cell viability was evaluated with the WST assay, as described in the "Experimental" section. Data are means ± SEM (n = 3). *Significant difference when compared with the vehicle-treated group ($P < 0.05$)

particles were stocked in a 5 mg/ml aqueous suspension. The stock solutions were suspended using a vortex mixer before use. Reagents used in this study were of research grade.

4.2. Animals

BALB/c male mice (8 weeks old) were obtained from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan), and were housed in an environmentally controlled room at 23 ± 1.5 °C with a 12 h light/12 h dark cycle. Mice had access to water and commercial chow (Type MF, Oriental Yeast, Tokyo, Japan). Mice were intravenously injected with nPt or snPt at 5 to 20 mg/kg body weight. The experimental protocols conformed to the ethical guidelines of the Graduate School of Pharmaceutical Sciences, Osaka University.

4.3. Cells

Mouse primary hepatocytes were isolated from BALB/c mice (Shimizu Laboratory Supplies Co.) by the collagenase-perfusion method (Seglen 1976). Isolated hepatocytes were suspended in Williams' E medium containing 10% fetal calf serum, 1 nM insulin, and 1 nM dexamethasone. Next, cell viability was assessed by Trypan blue dye exclusion. Cells that were at least 90% viable were used in this study. Cells were cultured in a humidified 5% CO_2 incubator at 37 °C.

4.4. Histological analysis

After intravenous administration of snPt, mouse livers were removed and fixed with 4% paraformaldehyde. Thin tissue sections were stained with hematoxylin and eosin for histological observation.

4.5. Biochemical assay

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using commercially available kits (WAKO Pure Chemical, Osaka, Japan), respectively. Interleukin-6 (IL-6) levels were measured with an ELISA kit (BioSource International, Camarillo, CA, USA). These assays were performed according to the manufacturer's protocols.

4.6. Cell viability assay

Cell viability was determined using WST-8 (Nacalai Tesque, Osaka, Japan), according to the manufacturer's protocol. Briefly, 1×10^4 cells/well were seeded on a 96 well plate at 37 °C overnight. After 24 h of treatment with snPt, WST-8 reagent was added to each well. The plate was incubated for 1 h at 37 °C and assessed at an absorbance of 450 nm by a plate reader. Obtained data were normalized to the control group, which was designated as 100%.

4.7. Statistical analysis

Data are presented as means ± SD. Statistical analysis was performed by student's t-test. $P < 0.05$ was considered statistically significant.

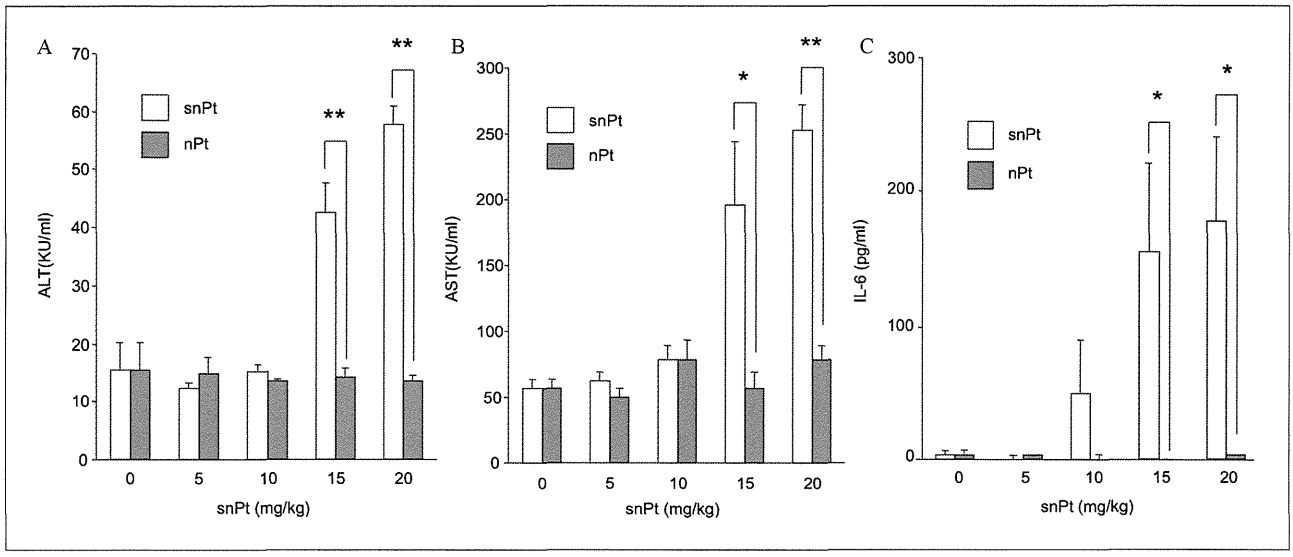

Fig. 5: Effect of particle size of platinum on liver injury. snPt or nPt was intravenously injected into mice at the indicated doses. Blood was recovered at 24 h after injection. Serum ALT (A), AST (B) and IL-6 (C) levels were measured. Data are means ± SEM (n = 3). *Significant difference between the snPt- and nPt-treated groups (*, $p < 0.05$, **, $p < 0.01$)

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