厚生労働科学研究費補助金（萌芽的先端医療技術推進研究事業）
分担研究報告書

7. *In vitro* 3次元微小腫瘍モデルの作製に関する研究

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研究要旨：本プロジェクトを推進する上で、新規な超音波分子バイオイメージングを検証するための「微小腫瘍モデル」の作製は必要不可欠な要素技術である。本研究では、アパタイト単結晶ファイバーから腫瘍モデル細胞の三次元足場を構築し、ラジアルフロー型バイオリアクターを用いて循環培養することにより微小腫瘍モデルの構築を指向した。

A. 研究目的

現在、骨再生や肝再生をはじめとして、細胞・増殖因子・足場材料の三要素を組み合わせて目的とする組織を再生する「ティッシュエンジニアリング」が注目されている。我々はこれまでに優れた生体適合性を有するアパタイト単結晶ファイバー（AF）を用いて細胞が内部へ侵入できる連通孔を持つ三次元的に培養できる細胞の足場材料（アパタイトファイバースキャフォルド；AFS）の開発に成功している。本研究では、このAFSを微小腫瘍モデル構築の足場材料としての適用を指向し、まず、第一段階の取り組みとしてAFSにヒト肝癌由来の肝細胞モデルであるFLC-4を播種してその三次元培養下で細胞増殖と形態について検討した。また、AFSを装填したラジアルフロー型バイオリアクター（RFB）を用いて三次元培養を行い、細胞の生育状態をモニターし、RFBの足場としてのAFSの実用性を検討した。

B. 研究方法

AFSは既報（M. Aizawa, et al., *Phosphorus Res. Bull.*, 17, 268-273(2004).）と同様にAFに質量比で10倍、20倍のカーボンビーズを添加した1mass%混合スラリーを調製し、成形後、1300℃で5h、水蒸気雰囲気下で焼成して作製した。なお、カーボンビーズ無添加で作製したスキャフォルドをAFS0とし、カーボンビーズを10倍および20倍添加したAFSをそれぞれAFS1000およびAFS2000とする。得られた焼結体の結晶相をXRDおよびFT-IRにより同定し、微細構造をSEMにより観察した。AFSの生物学的評価はFLC-4を用いて行なった。各基材における細胞増殖性の比較のため5.0×10^5 [個/cm^3]の細胞密度で1 cm^3ずつAFS2000, AFS1000, AFS0, Controlに播種して28日間培養し、DNA量の測定、形態観察を行った。また、RFBを用いて三次元培養を行い、培地中のグルコース量、乳酸値、pHから細胞の生育状態のモニターと28日後の形態をSEMにより観察した。

（倫理面の配慮）

ヒトES細胞など特に倫理面を考慮する実験系を使用していない。

C. 研究成果

XRDよりAFSの結晶相は*c*軸配向した水酸アパタイト（Hap）単一相であることがわかった。SEMによる微細構造の観察から、AFS0とAFS2000の気孔径を比較すると約5μmから250μmに拡大しており、気孔同士の連通も確認された。このような細孔構造は細胞が内部まで侵入でき、三次元培養に適していると考えられる。図1に細胞増殖の結果を示す。なお、この図は細胞数をそのDNA量で規定している。どの基材においても細胞は良好に増殖した。特に、AFSグループの増殖性が高く、その中でもAFS2000, AFS1000, AFS0の順に良好な増殖を示した。これは連通孔が大きいほど細胞が侵入しやすく、培養面積も広いということを示している。SEM観察より、細胞がポアに入り込んでいる様子が観察され、ポア以外の場所ではシート状になって細胞が増殖していた。

また、RFBを用いた細胞の生育状態のモニター結果より、グルコース量が減少していることから細胞がそれを栄養分として消費していることがわかった。一方、乳酸値は上昇しており、細胞がグルコースを代謝して産生したものと考えられる。これらの結果は、今回モニターした28日間で細胞が良好に増殖していることを示しており、本スキャフォルドはRFBを用いた三次元培養の足場としても有効であると考えられる。

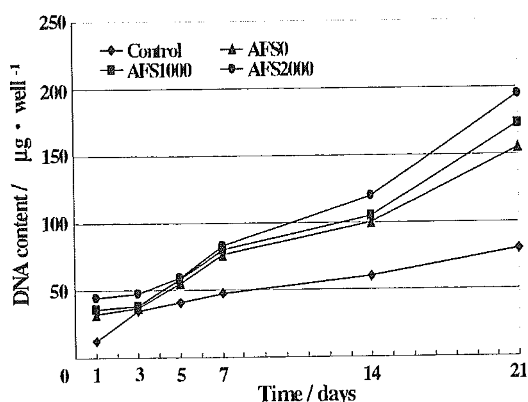

Fig.1 Growth curves of the FLC-4 cells in AFSs, together with Control.

D. 考察

本研究で試用したAFSは微小腫瘍モデル構築の足場材料として有効であると考えられる。

E. 結論

本AFSは細胞の三次元的培養が1ヶ月以上の長期にわたり可能であったが、これはAFSのもつ特異な微細構造、すなわち、細胞の進入可能なマクロ気孔と培地などの栄養物質の浸潤が可能なミクロ気孔の存在によるものと考えられる。また、アパタイトの結晶面のひとつであるa面を多く露出していることから、生体吸収性も備えており、本プロジェクトの微小腫瘍モデル構築に向けた足場材料として有効であると結論できる。今後は最適な細孔などについて検討を進める。

F. 健康危険情報

報告書にまとめて記載。

G. 研究発表

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H. 知的財産権の出願状況

1. 特許取得

1-1) 相澤 守、松浦知和、特願2005-079350・バイオリアクター・学校法人明治大学

1-2) 相澤 守、松浦知和、特願2005-079350・肝細胞の培養・学校法人明治大学

厚生労働科学研究費補助金（萌芽的先端医療技術推進研究事業）
分担研究報告書

8. 超音波造影剤検定のための3次元還流培養腫瘍モデルの作成

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協力研究者　政木隆博　国立感染症研究所　リサーチレジデント
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研究要旨：超音波造影剤検定のための3次元還流培養腫瘍モデルを、ラジアルフロー型バイオリアクター（RFB）を用いて作成した。癌細胞、肝細胞、血管内皮細胞をRFBで共培養し、血管様構造を持つ腫瘍あるいは肝臓オルガノイドが作成できた。また、超音波観察用のプラスチック製RFBも試作した。超音波透過性は良好で、RFBでの3次元腫瘍を超音波で観察することが可能となった。今後このモデルを用いて、超音波造影剤であるマイクロバブルの生体内特性、超音波による音響特性を検討する。

A. 研究目的

超音波造影剤（マイクロバブル）の新規開発のためには、マイクロバブルの生体での、①安定性（肺や肝臓でのトラップなど）、②血管透過性、③生体内代謝・毒性、④CD147をターゲットとした腫瘍集積性、⑤超音波による描出性などの検討が必要である。新規界面活性剤を用いて種々の条件（大きさ、粘性）のマイクロバブルを作製し、その超音波描出性をすべて動物実験で行うことは、困難である。このため、小型ラジアルフロー型バイオリアクター（RFB）を用いて、癌細胞を3次元的な腫瘍塊として培養し、そこに開発したマイクロバブルを還流して超音波での描出ができるか検討することとした。平成17年度は、①ヒト肝臓癌細胞株あるいはマウス不死化肝細胞株とマウス内皮細胞株を共培養し、3次元腫瘍塊あるいは肝臓オルガノイドの作成を試みた。また、②超音波観察のための、プラスチック製小型RFBを試作した。

B. 研究方法

①3次元ヒト肝癌モデルと肝臓オルガノイドの作成

ヒト肝細胞癌株FLC-5とマウス不死化内皮細胞M1、不死化伊東細胞A7をRFB内で共培養した。培養担体として、多孔質セルロースビーズを用いた。肝臓オルガノイド作成は、相澤の開発したハイドロキシアパタイトファイバー（HAF）カラムをRFBに装填し、マウス不死化肝細胞IMH-4とM1およびA7を共培養した。

②超音波観察用プラスチック（TPX）製RFBの設計と試作

超音波で3次元腫瘍モデルや臓器モデルを観察するには、従来のステンレス製RFBでは不可能であるため、5ml容量のTPX製RFBを設計・試作した。内部には溶解性スカフォードHAFカラムを装填した。

C. 研究結果

①3次元ヒト肝癌モデルと肝臓オルガノイドの作成

肝癌モデルは、還流側（Apical側）より内皮細胞、伊東細胞、FLC-5と配列された（図1）。また、内皮細胞には口径 100-200μmの小孔が出現した（図2）。肝臓モデルでは、3種類の細胞を2週間共培養したところ、細胞が増殖・配列し、組織化した（図3）。以上から、形態学的に3次元腫瘍モデルと肝臓オルガノイドをRFB内で作成することができた。

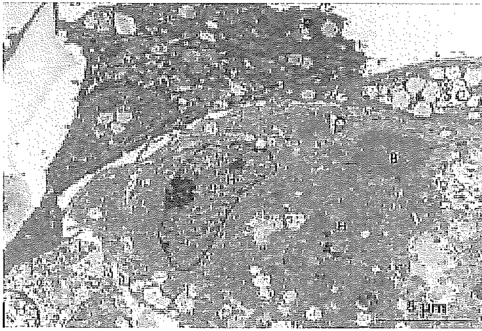

図1:3次元腫瘍モデル

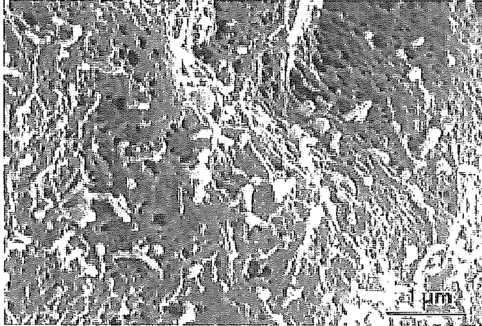

図2:内皮細胞の小孔

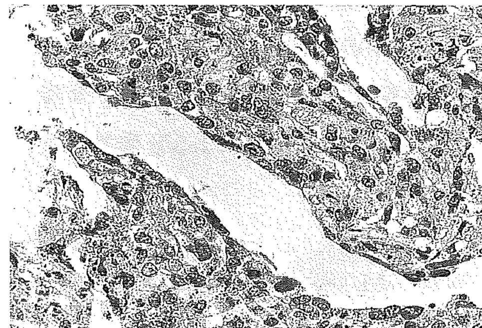

図3:肝臓オルガノイド

②超音波観察用プラスチック(TPX)製 RFB の設計と試作

TPX 製 RFB カラムを水槽にいれ、超音波を底面にあてると、ゼラチンで固めたガラスビーズを観察することができた。本システムでマイクロバブルを描出し、その腫瘍集積性を観察することが可能と考えられた(図4, 5, 6)。

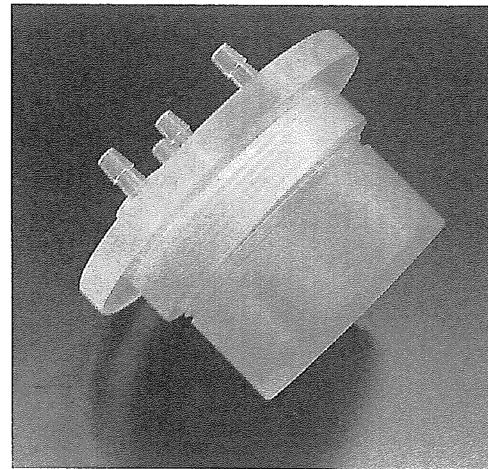

図4:プラスチック製 RFB

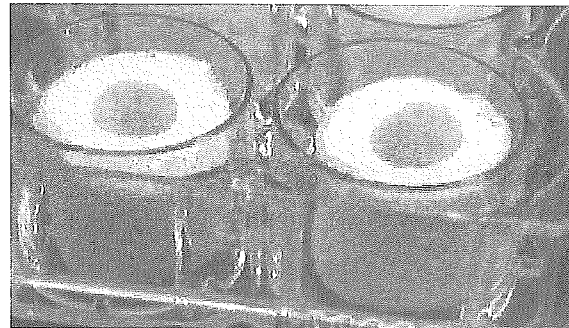

図5:溶解性 HAF カラム

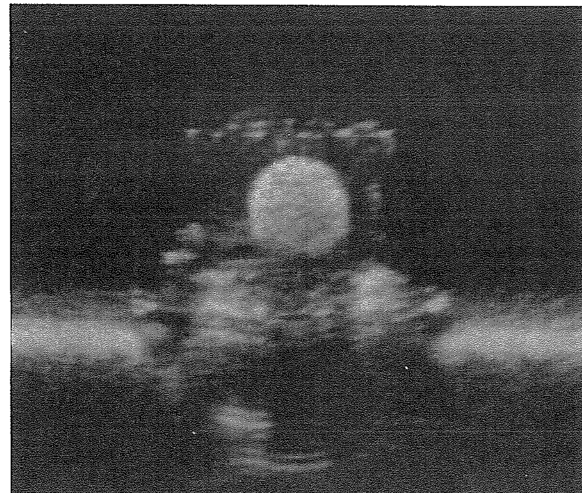

図6:超音波による描出

D. 考察

超音波造影剤としてのマイクロバブルの生体内特性のシミュレーションと超音波での観察のため、RFB を用いた3次元腫瘍モデルと肝臓オルガノイドが作成できた。今後試作したマイクロバブルを還流し、その音響特性や腫瘍集積性をこのモデルで検討し、in vivo への応用をめざす予定である。

E. 結論

TPX 製 RFB 培養システムを用いた3次元癌モデルは、超音波造影剤としての新規マイクロバブルの特性を検討する上で有用である。

F. 健康危険情報

G. 研究発表

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3) 斉藤勝也、松浦知和、政木隆博 他:3次元還流共培養下でのオレアミド投与による再構築肝組織の形態変化(類洞内皮細胞の篩板状小孔について) 第41回日本肝臓学会総会 2005年6月17日

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H. 知的財産権の出願・登録状況

特許出願

ガス混合装置及びガス混合法

(出願日 2006年2月27日、特願2006-050548)

厚生労働科学研究費補助金(萌芽的先端医療技術推進研究事業)
分担研究報告書

9. 動物実験:腫瘍動物モデルの開発に関する研究

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研究要旨:本年度は、ヒト癌を用いた移植動物モデルおよびマウスの転移細胞系を用いた腫瘍動物モデルの基礎的検証を行った。また、癌の浸潤・転移における腫瘍細胞と間質細胞の相互作用に重要な役割を演じている細胞外マトリックステネイシンにも着目し、動物腫瘍モデルの解析・検証を行った。

A. 研究目的

本分担研究の主目的は、現在開発中のマイクロバブルを用いた生体観察を行うための腫瘍動物モデルを確立することである。

実際の癌における CD147 の発現の強弱は癌の分化度や癌の種類によって様々である。その理由は、癌の浸潤・転移過程においてその周囲の間質細胞との相互作用が大きな影響力を持っており、癌の成長に必要な分子の遺伝子発現に多大な影響を持っていると考えられるからである。そのため、*in vivo* 実験系においては、癌の種類とホストの関係が重要であるので、ヒト細胞とヌードマウスといった系のみならず、マウス腫瘍を用いた転移モデルでの実験系を樹立することを目的にこれらの基礎的解析を行った。

B. 研究方法

1. ヒト癌細胞の移植による腫瘍動物モデル系の開発:

1) 移植に用いるヒト癌細胞株におけるCD147の発現の有無の検討:

A431(類表皮癌)、A375(メラノーマ)、A549(肺癌)、HLC1(肺癌)および CMT315(マウス乳癌細胞株)の培養下での CD147 の発現を、ALEXA488 標識抗 CD147 単抗体 12C3 を用いた免疫染色によって検討した。

2) 性質の違う癌細胞を用いた移植モデル:実際の臨床診断において、標的とする癌は様々な特性を持っており、その予後診断は容易ではない。そこで性質の異なる細胞間におけるCD147を含む種々分子マーカーの動態を捉えることは重要なことである。今回は、抗がん剤感受性抗がん剤(シスプラチン)に対する感受性の違う卵巣癌細胞2008(感受性細胞)およびC13/2008(耐性細胞)を正常ヌードマウスおよびテネイシン欠損ヌードマウスの背部皮下にそれぞれ移植して、固形腫瘍を形成させ、そのテネイシンの発現を免疫組織学的に解析した。

2. マウス転移腫性瘍細胞の移植による腫瘍動物モデル系の開発:

本研究の主目的は、標的分子を用いた生体内イメージングによって癌の超早期診断を行うことである。そのため、腫瘍の浸潤・転移などの病態の経過観察ばかりでなく、転移巣などの検出やどのくらいの大きさの腫瘍であれば生体内で検出できるのかを検証できる腫瘍動物モデルの開発が重要である。これらの検討を行うためには、マウス腫瘍細胞を用いた移植系が色々な点で人の癌の病態を反映していると考えられる。そこで、本年度は、以下の移植実験を行った。

1)マウス乳癌細胞株を背部皮下にマウス乳癌細胞を移植し、4週間腫瘍を成長させた。その後、肺や肝臓に転移した転移腫瘍を病理組織学的に解析した。

2)本実験で用いている抗 CD147 単抗体 12C3 はヒト特異的な抗体であるので、マウスの CD147 を認識する抗体の作成を開始した。

C. 研究結果

1. ヒト癌細胞の移植による腫瘍動物モデル系の開発

1) 移植に用いるヒト癌細胞株におけるCD147の発現の有無の検討:用いた12C3抗体はヒト特異的である。検討したヒト細胞株は、発現の強弱があるもののすべて陽性であった。しかし、マウス腫瘍細胞は陰性であった。これらの細胞のうち、培養下でテネイシン非産生株(A431とA549)と培養下でテネイシン産生株(A375とHLC1)における染色パターンに違いが見られた。後者の方は、前者よりも強い反応が細胞の辺縁に観察されるが、前者の細胞では、弱い反応が細胞表面全体に散在的に観察された。

2)性質の違う癌細胞を用いた移植モデル:

シスプラチン感受性 2008 は、移植後1週間後から腫瘍形成を確認でき、4週目では直径1.5cm ほどに成長した。一方、シスプラチン耐性C13の成長は、かなり遅く同程度の大きさになるのに8週間ほどかかった。それぞれの腫瘍に

おけるテネイシン発現を免疫染色で検討したところ、2008 腫瘍では、ホスト間質細胞由来のテネイシンとともに腫瘍自体の産生するテネイシンが癌巣内外に沈着して見られた。それに反し、C13 腫瘍では、ホスト間質由来のテネイシンが観察されるものの、腫瘍由来のテネイシンは、一部を除いて殆ど検出することが出来なかった。ヌードマウスに移植した両腫瘍の免疫染色では、2008 は腫瘍自体が強くテネイシンを発現していることが分かったが、C13 腫瘍では、全くテネイシンが検出できなかった。

2．マウス転移腫性瘍細胞の移植による腫瘍動物モデル系の開発：

1）背部皮下にマウス乳癌細胞を移植し、4週間腫瘍を成長させ後、これらマウスの各臓器を肉眼および顕微鏡レベルで観察したところ、肺および肝臓に転移腫瘍が観察できた。これらの腫瘍は、テネイシンを発現していた。肺における転移腫瘍の大きさは、直径500 μmから1mm位のものが散在性に存在していた。肝臓における転移腫瘍は、比較的大きく成長していたがそれでも3mm程度であった。

2）マウスの抗体は、現在作成中であるが、十分な抗体価を示す抗体は得られていない。今後継続していく予定である。

D. 考察

1．ヒト癌細胞の移植による腫瘍動物モデル系の開発：

1）移植に用いるヒト癌細胞株におけるCD147の発現の有無の検討：培養下でテネイシン非産生細胞株も、皮下移植によって固形腫瘍を形成し、テネイシンを発現することは知られている。また、この発現調整は、ホストの間質環境が重要な役割を担っているので、間質細胞との共培養によってこの発現パターンがどのように変化するかは興味あるところである。今後、この点に視点をおいてモデルを樹立する予定である。

2）性質の違う癌細胞を用いた移植モデル：抗がん剤に対する感受性の違いによってテネイシンの発現が異なっていることが判明した。このことからも、腫瘍細胞と癌の微小環境の関係が、多くの分子の発現に影響することが示唆され、上記の実験結果と共に、この相互作用に重点を置いて動物モデルを樹立する必要があると考えられる。

2．マウス転移腫性瘍細胞の移植による腫瘍動物モデル系の開発：

マウスの転移モデルでは、皮下腫瘍（移植原発腫瘍）から自然転移によって形成された腫瘍細胞塊であり、その微小環境もヒトのがん組織形成のモデルとなりうると考える。更に、超早期診断を目指す観点からも、これらの腫瘍の大きさからも極初期のヒト腫瘍のモデルとして利用価値が高いと考えられる。一方、マウスのCD147 を認識する抗体の作成によってその動態が検証されることが重要であるのでこの抗体の作成を急ぎたい。

倫理面への配慮

本研究は現在までのところ、動物実験による本プロジェクトの検証用のモデル開発が目的である。動物を用いた実験は全て全身麻酔下に行っており苦痛を伴うものではない。本研究における動物実験は財）動物繁殖研究所の動物実験指針に則って行われた。

E. 結論

本年度行った動物実験からは、生体における本分子の発現は、環境から影響を受けることが予測できる。そのため、動物腫瘍モデルの樹立に際して、ホスト組織の微小環境の影響を十分考慮する必要があると考える。今後、癌とその間質細胞との相互作用を、上手く利用できる動物腫瘍モデルの樹立を目指して行く予定である。

F. 健康危惧情報

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H. 知的財産権出願・登録状況

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

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Relationship between expression of drug-resistance factors and drug sensitivity in normal human renal proximal tubular epithelial cells in comparison with renal cell carcinoma

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Abstract. The relationship between the expression level of putative drug resistance factors and sensitivity to anticancer drugs in human normal Renal Proximal Tubule Epithelial Cells (RPTEC) and 3 kinds of renal cell carcinoma (RCC) cells, VMRC-RCW (RCW), OS-RC-2 (OS2), TUHR14TKB (14TKB), was examined. RPTEC exhibited high expression of P-glycoprotein (Pgp), γ-glutamyl cysteine synthetase (γGCS) and cis-diamminedichloroplatinum (II) (CDDP) resistance-related gene 9 (CRR9), low expression of vacuolar ATPase (V-ATPase) and no expression of multidrug resistance-associated protein 1 (MRP1). 14TKB exhibited high expression of γGCS and CRR9, low expression of Pgp and V-ATPase, and no expression of MRP1. OS2 showed high expression of CRR9, low expression of Pgp, γGCS and MRP1, and no expression of V-ATPase. RCW exhibited high expression of Pgp, MRP1 and CRR9 and low expression of γGCS and V-ATPase. The level of expression of the resistance factors varied among the cells. GST activity and GST-π expression level of each cell were correlated, and there were high levels in OS2 and RPTEC. When the cytotoxicity of anticancer drugs against each cell was measured at 96 h, the sensitivity to CDDP and Doxorubicin (DXR) in RPTEC and RCW was lower than that in the other cells. Sensitivity to DXR was enhanced by treatment with the Pgp inhibitor, Verapamil, in proportion to the Pgp expression level, and the sensitivity to CDDP was increased by the γGCS inhibitor, Buthionine sulfoximine, in proportion to the γGCS expression level (corresponding to GSH content). Although a significant increase in sensitivity to CDDP was not observed by treatment of RCC with the V-ATPase inhibitor, Bafilomycin, the sensitivity to DXR in Bafilomycin-treated cells increased about 2-fold. However, no relation between drug sensitivity and V-ATPase expression was observed. The features (such as degree of resistance) varied among the RCC cell lines manifesting many resistance factors or to the contrary, lacking or having lowered resistance factors in comparison with normal cells. Therefore, it is necessary in clinical cancer chemotherapy to determine and measure the level of expression of each resistance factor in respective tumor tissue.

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Key words: drug resistant factor, P-glycoprotein, multidrug resistance-associated protein, γ-glutamyl cysteine synthetase, glutathione, CDDP resistance-related gene 9, glutathione S-transferase, doxorubicin, cisplatin, renal cell carcinoma, normal proximal tubule epithelial cells

Introduction

The mechanism of drug resistance to various anticancer drugs has been reported, and many basic studies have been conducted in order to overcome this problem. Accordingly, the expression of genes or proteins related to the mechanism of resistance in the cancer of each individual should be investigated.

In general, renal cell carcinoma (RCC), which is derived from renal tubular epithelial cells, shows resistance to cancer chemotherapy. In the tubular epithelium of the kidney, various kinds of transporters are expressed for metabolite resorption (1) and it is predicted that RCC reflects the character of the tubular cells. Since it appears that these transporters also transport various drugs including anticancer drugs, it is predicted that natural drug tolerance, which differs from acquisition resistance, strongly protects the RCC. However, a regimen for effective systemic chemotherapy of RCC has not yet been established.

The mechanism by which resistance appears, and the phenomenon of multidrug resistance (MDR) which simultaneously causes clinical resistance to various anticancer drugs have gained attention, and research into the basic mechanism has made positive advances. Overexpression of P-glycoprotein (Pgp) is observed in many MDR cells (2,3). Pgp, a membrane protein with a molecular weight of about 170 kDa, belongs to the ABC (ATP binding cassette) transporter superfamily that exports many kinds of anticancer drug differing in their structure and pharmacological action from the cell using the hydrolysis energy of ATP. Subsequent research

has shown that Pgp has appeared in various normal tissue cells (4,5). Pgp which appears strongly in the lumen brush border membrane of the proximal tubule is mainly involved in transmission in urinary excretion of comparatively hydrophobic cationic compounds, such as anthracycline drugs, vinca alkaloid drugs, digoxin, cyclosporin and steroid hormones. This appears to be one mechanism of natural resistance in renal cancer. However, the mechanism of MDR can not be explained by Pgp alone. MRP (multidrug resistance-associated or -related protein), which is a membrane protein with a molecular weight of 190 kDa, has been found in many resistant cells with multidrug resistance unrelated to Pgp (non-Pgp-mediated MDR) (6-9). Although the amino acid sequence of MRP has only 17% homology with Pgp, both Pgp and MRP exhibit resistance to common drugs such as anthracycline and vinca alkaloid (6). Moreover, since MRP also transports organic anions, unlike Pgp, it is known that MRP is a transportation protein that acts via conjugation of the drug with glutathione, glucuronic acid or sulfate (6). Since MRP is localized in the basal membrane on the side of the tubular epithelial cell, it appears to be related to the efflux of the drug reabsorbed from the uriniferous tubular lumen into the blood. The anticancer drug, Doxorubicin (DXR), suppresses the biosynthesis of both DNA and RNA by inhibiting both DNA and RNA polymerase reactions due to the formation of a complex between DXR and the DNA of tumor cells, thus enabling DXR to exhibit an antitumor effect (10). However, DXR is pumped out by Pgp and MRP molecules from the intracellular to the extracellular environment, and both molecules are involved in drug resistance. An anticancer drug such as cis-diamminedichloroplatinum (II) (CDDP) that does not become a substrate of Pgp and MRP also exists. Since CDDP is conjugated with glutathione (11) in the metabolic process and MRP transports many GSH conjugates, it can be predicted that MRP is associated with the CDDP efflux pump. However, cell lines with high levels of MRP expression do not show cross tolerance to CDDP, and the CDDP-resistant cell lines do not always overexpress MRP (6,12).

An attempt was made to use Verapamil as a treatment to overcome the effects of MDR since it was known that this drug combined with the drug binding site of Pgp and inhibited efflux of anticancer drugs from the cell to the extracellular environment by preventing Pgp function (13-17).

GSH, which acts as an *in vivo*-SH buffer has various functions and it is important for the maintenance of homeostasis. The functions of GSH that relate to drug tolerance (18,19) are as follows: i) GSH conjugation, ii) reduction of peroxide produced by the drug, and iii) DNA repair. In particular, GSH is related to detoxication of CDDP by non-enzymatic conjugation, and anthracycline drug conjugation through GST and transportation by MRP1, thereby lowering the intracellular drug level. GSH biosynthesis is regulated by γ-glutamyl cysteine synthetase (γGCS) as a rate-limiting enzyme. Lowering of the intracellular GSH content by the γGCS activity inhibitor, buthionine sulfoximine (BSO), overcomes drug resistance through the suppression of GSH conjugation and the subsequent increase in intracellular anticancer drug level in MRP-expressing cells (20,21).

Vacuolar ATPase (V-ATPase), which is expressed at a high level in tubule epithelial cells of the kidney and osteoclasts, is localized in the cell membrane and is involved in extracellular acidification (22). V-ATPase is an H^+ transportable ATPase that plays a major role in the formation of an internal acidic environment in the subcellular organellae (Golgi body, lysosome, secretion granule, coated vesicle, endosome, etc.) belonging to the intracellular membrane system of the eukaryote. The acidic internal environment of the subcellular organella and the division of the extracellular environment formed by V-ATPase are essential for the concentration of neurotransmitters, hormones and ions and for the degradation of proteins and lipids. As a result of a recent experiment using human throat carcinoma KB cells (22), the involvement of high-level expression of V-ATPase was indicated as a new mechanism of CDDP resistance (23,24). This mechanism appears to act via the suppression of intracellular accumulation of the basic drug in the alkalinized cytoplasm by high-level expression of V-ATPase that promotes cross-linking of DNA and CDDP under acidic conditions. However, it is uncertain whether or not Bafilomycin, a macrolide antibiotic, is a specific inhibitor that can modify this mechanism and reduce the resistance.

CDDP resistance-related gene 9 (CRR9) seems to be the membrane transport protein of CDDP that is pumped out of the cells, though details of the mechanism remain unclear.

It has been shown that glutathione S-transferase (GST) is one of the molecules that are closely related to drug delivery and detoxication (25-27). GST is a multifunctional enzyme that consists of molecular species that it mainly transfers to GSH in drugs and various endogenous nucleophilic compounds, thus preparing for GSH conjugation. GST species mainly exist in the cytoplasm, and these molecular species are classified into the α, μ, π and ø classes (28-30) by their enzymologic properties, N-terminal amino acid sequence and immunological properties. It has been reported that GST-π, which shows high level expression in carcinoma tissue, is closely related to anticancer drugs (28,29) and the GST-π expression level. Moreover, it exhibits an inverse correlation with the drug sensitivity of liver cancer and colon cancer (27,30,31).

We examined here the sensitivity to DXR and CDDP and the mRNA expression levels of Pgp, MRP, γGCS, V-ATPase and CRR9 by RT-PCR in normal human renal proximal tubular epithelial cells, which constitute a genesis of RCC, and 3 kinds of human RCC-derived cell lines, VMRC-RCW (RCW), OS-RC-2 (OS2) and TUHR14TKB (14TKB). Moreover, the change in sensitivity caused by the inhibitor of each resistance factor was compared in each cell line. In addition, we investigated whether or not the expression level of GST-π was related to the resistance to anticancer drugs in RCC.

Materials and methods

Cell lines. A human renal proximal tubular epithelial cell (human renal proximal tubule epithelial cells, RPTEC) was obtained from Takara, RCW was from Health Science Research Resources Bank (Tokyo, Japan), OS2 and 14TKB from Riken. RPTEC was cultivated in renal epithelial cell basal medium including 10 ng/ml human recombinant epidermal growth factor, 5.0 μg/ml insulin, 0.5 μg/ml hydrocortisone, 0.5% FBS (fetal bovine serum), 0.5 μg/ml epinephrine, 6.5 ng/ml

triiodothyronine, 10 μg/ml transferrin, 50 μg/ml gentamicin and 50 ng/ml amphotericin-B at 37˚C in 5% CO_2. RCW, 14TKB and OS2 were cultured in RPMI-1640 containing 10% FBS at 37˚C in 5% CO_2.

Materials. CDDP was purchased from Bristol-Myers Squibb (Tokyo, Japan), DXR was obtained from Kyowa Hakko Kogyo (Tokyo). BSO, GSH, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazorium bromide (MTT), *o*-phthalaldehyde (OPT), 1-chloro-2,4-dinitrobenzene (CDNB), 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (BCIP/ NBT) were obtained from Sigma-Aldrich Japan (Tokyo). Ex Taq DNA polymerase and M-MLV Reverse Transcriptase were purchased from Takara (Tokyo). Bafilomycin A was from Wako (Osaka, Japan). FuGENE6 was obtained from Boeringer Mannheim (Tokyo). Trisol LS was purchased from Invitrogen (Tokyo). All other chemicals were of analytical grade.

Assay of GST activity and GSH content. GST activity was measured at 340 nm (ε = 9,600) in 1 mM CDNB and 1 mM GSH at 37˚C for 15 min (32). GSH content was measured in GSH conjugated with OPT by the method of Cohn *et al* (33).

Cytotoxicity of CDDP and DXR. To assess the growth inhibitory effect of CDDP and DXR, viable RPTEC and RCC cells ($2x10^4$) were cultured continuously for 96 h in a 48-well culture plate (Corning Coster) with 0.5 ml of CDDP or DXR containing growth medium at graded equivalent concentrations of each drug in the presence or absence of 5 μM Verapamil, 0.3 mM BSO or 1 μM Bafilomycin. The cultivation was carried out after 24-h treatment of cells with BSO. After incubation, viable cells were determined with the colorimetric assay using MTT as described previously (34), and the results were expressed as previously (35-42).

Expression of Pgp, MRP1, γGCS, V-ATPase and CRR9. Total RNA of each cell was extracted using Trisol LS reagent. The cDNA was prepared by reverse transcription using the total RNA, and the expression level of Pgp, MRP, γGCS, V-ATPase and CRR9 was measured by PCR using the obtained cDNA as a template. Each factor was compared with ß-actin as an internal standard. Primer for each factor was used as follows; ß-actin (254 bp): AACACCCCAGCC ATGTAC (sense), ATGTCACGCACGATTTCC (antisense); Pgp (161 bp): AAAAAGATCAACTCGTAGGAGTG (sense), GCACAAAATACACCAACAA (antisense); MRP1 (990 bp): AATGCGCCAAGACTAGGAAG (sense), ACCGGAGGAT GTTGAACAAG (antisense); γGCS (521 bp): TGAGATTTA AGCCCCCTCCT (sense), TGCGATAAACTCCCTCATCC (antisense); V-ATPase (500 bp): ATGTATGAGCTGGTGG AGGTGGGCC (sense), TTGACGTGCAGGCCATACTTG CACC (antisense). The amount of expression of each factor was quantified by densitometry.

Detection of GST-π. Cell lysate extracted with 2% sodium dodecylsulfate was analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis on 12.5% polyacrylamide gel as previously reported (36,37,42), followed by Western blotting onto nitrocellulose filters. After immunoreaction using murine anti-human GST-π antibody (x1/1,000) as the primary antibody and anti-mouse IgG-alkaline phosphatase conjugate as the secondary antibody (x1/1,000), GST-π band was visualized with BCIP/NBT.

Table I. Cell growth activity, GSH concentration and GST activity in PRTEC, RCW, OS2 and 14TKB cells.

	Doubling time (h)	GSH concentration (nmol/mg protein)	GST activity (nmol/min/ mg protein)
RPTEC	24	22±2.9	21±4.1
14TKB	60	120±14.1	7±2.6
OS2	32	4±1.9	70±6.7
RCW	48	25±3.2	10±3.3

Cell growth activity was expressed as doubling time of cells. GSH content was measured in GSH conjugated with OPT by the method of Cohn *et al* (33). GST activity was measured at 340 nm (ε=9,600) in 1 mM CDNB and 1 mM GSH at 37˚C for 15 min (32). Results are means ± SD (three independent experiments).

Results and Discussion

Cell growth rates, exhibited as the doubling time of RPTEC, 14TKB, OS2 and RCW as a characteristic of RCC cell lines, were 24, 60, 32 and 48 h respectively (Table I). All RCC cell lines examined showed slow growth in comparison to RPTEC. This lowering of the cell growth ability following carcinogenesis was suggested to be an element underlying the slowing of the appearance of symptoms, and supported by the same report (7).

The drug sensitivities of RPTEC, 14TKB, OS2 and RCW were compared (Fig. 1). As shown in Table II, the IC50 values of CDDP for RPTEC, 14TKB, OS2 and RCW were 3.0, 3.2, 1.1 and 3.5 μg/ml respectively, and those of DXR for RPTEC, 14TKB, OS2 and RCW were 2.1, 1.1, 1.0 and 2.0 μM respectively. OS2 was the most sensitive to CDDP and DXR in all cells and 14TKB exhibited high sensitivity to DXR. It was shown that the sensitivity to either drug in RPTEC was low (high IC50 value) in relation to the level in either of the RCC cell lines.

The appearance of tumor resistance to an anticancer drug causes failure of cancer therapy. RCCs have been classified as marginal or poorly responsive in terms of the chemotherapeutic effect because of the natural resistance of the tumor cells (1,43-46). In fact, the IC50 values of DXR in human hepatoblastoma HepG2 cells, human colon cancer HT29 cells and human leukemia K562 cells were 0.4, 1.0 and 0.1 μM (35,47) respectively, compared with 1.0-2.1 μM for the RCC cell lines examined in this report (Table II). This result is derived from the multiple transporters that act to export foreign matter, metabolites and excreta from the intracellular to the extracellular environment. These transporters are expressed and function in the tubular epithelial cells of the kidney. However, the mechanism and the clear reverse of resistance has rarely been reported. Since RCCs possess various properties with slow progress, rapid transition and

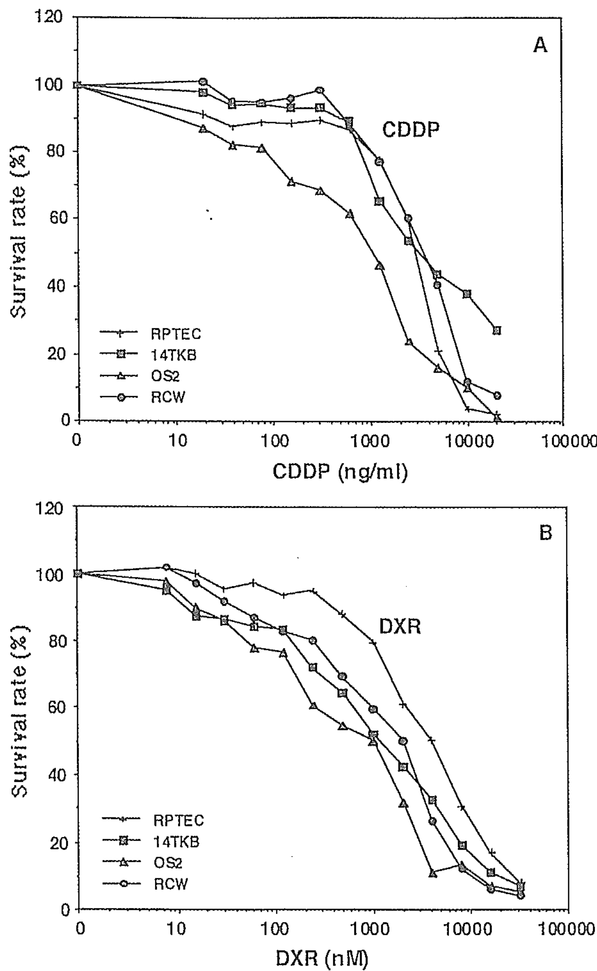

Figure 1. Cytotoxicity of CDDP (A) and DXR (B) against RPTEC, 14TKB, OS2 and RCW cells. Viable RPTEC and RCC cells (2×10^4) were cultured continuously for 96 h with 0.5 ml of CDDP or DXR containing growth medium at graded equivalent concentrations of each drug. Viable cells were determined with the colorimetric assay using MTT.

Table II. IC50 values of CDDP and DXR for RPTEC, 14TKB, OS2 and RCW cells.

	IC50	
	CDDP (μg/ml)	DXR (μM)
RPTEC	3.40	2.10
+ Ver	2.10	0.43
+ BSO	0.59	4.00
14TKB	3.20	1.10
+ Ver	2.50	0.23
+ BSO	1.50	0.71
+ Baf	3.30	1.05
OS2	1.10	1.00
+ Ver	1.05	0.41
+ Baf	1.00	0.41
RCW	3.50	2.00
+ Ver	4.20	0.43
+ BSO	1.50	0.71
+ Baf	5.30	1.05

+Ver, Co-treatment of cells with 5 μM Verapamil, an inhibitor of Pgp. +BSO, Cytotoxicity assay after 24-h treatment of cells with 0.3 mM buthionine sulfoximine (BSO), an inhibitor of γ-glutamyl cysteine synthetase. +Baf, Co-treatment of cells with 1 μM Bafilomycin, an inhibitor of V-ATPase. Results are means ± SD (three independent experiments).

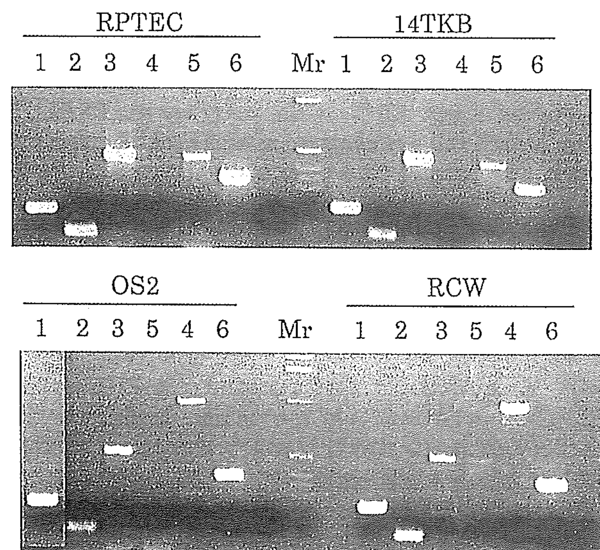

Figure 2. Analysis of expression of drug resistant factor (Pgp, γGCS, MRP1, V-ATPase and CRR9) in RPTEC, 14TKB, OS2 and RCW cells by RT-PCR. Lane 1, ß-actin (254 bp); lane 2, Pgp (161 bp); lane 3, γGCS (521 bp); lane 4, MRP1 (990 bp); lane 5, V-ATPase (500 bp); lane 6, CRR9 (387 bp).

malignancy (7,46,48,49), RCC has become an interesting tumor for use in cell biological research.

The difference in expression of mRNA of the factors related to drug resistance (Pgp, MRP, γGCS, V-ATPase and CRR9) was compared between RPTEC, as one of the normal cells, and RCC cell lines. The expression pattern of each factor, measured in RPTEC and RCCs by the RT-PCR method, is shown in Fig. 2, and the expression rate of each factor compared with ß-actin as an internal standard is shown in Fig. 3. The relationship between the expression of drug resistance factors and sensitivity was compared in RPTEC and RCCs. High expression of Pgp, γGCS and CRR9 and low expression of MRP1 and V-ATPase were observed in RPTEC. It was reported that MRP1 and V-ATPase were expressed in normal renal tubular cells (1,22). However, it was not possible to compare the expression level in RPTEC and the reported cells. One of the RCC cell lines, RCW, expressed a large amount of Pgp, γGCS, MRP1 and CRR9, but, apart from MRP1, these factors were expressed to a smaller extent than in RPTEC. A higher drug sensitivity was expected in RCW than in RPTEC. IC50 values of CDDP and DXR were 3.4 μg/ml and 2.1 μM for RPTEC, and 3.5 μg/ml and 2.0 μM for RCW respectively (Table II), and the sensitivity

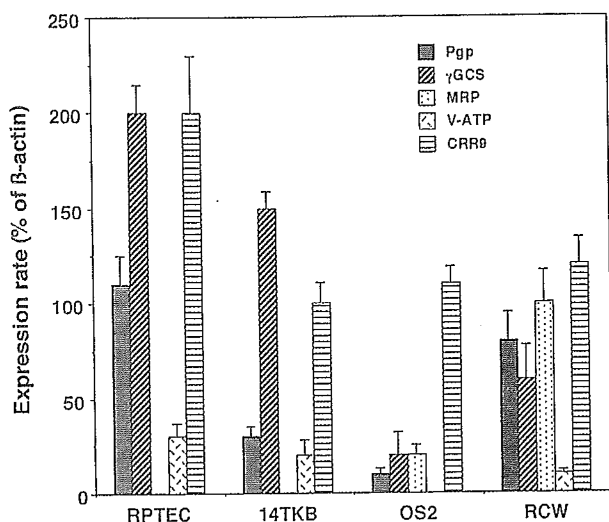

Figure 3. Expression level of drug resistant factors (Pgp, γGCS, MRP1, V-ATPase and CRR9) in RPTEC, 14TKB, OS2 and RCW cells. The amount of expression of each factor quantified by densitometry. The expression level was expressed as % of ß-actin (internal standard). Results are means ± SD (three independent experiments).

to both drugs in RCW was approximately the same as in RPTEC. It was suggested that RCW maintained the characteristics of RPTEC during carcinogenesis. On the other hand, the Pgp expression level in 14TKB was extremely low in comparison with the level in RPTEC, although other resistance factors in 14TKB were expressed at the same level as in RPTEC. IC50 values of CDDP and DXR in 14TKB were 3.2 μg/ml and 1.1 μM respectively (Table II). 14TKB exhibited high sensitivity to DXR compared with RPTEC. OS2 also expressed a low level of Pgp and the IC50 values of CDDP and DXR in OS2 were 1.1 μg/ml and 1.0 μM respectively. These results suggested that Pgp exhibited strong resistance to DXR, but CDDP was not a substrate of Pgp.

Accordingly, in order to confirm the relation of Pgp to drug sensitivity, the effect of the Pgp inhibitor, Verapamil, on the cytotoxicity of CDDP and DXR was examined. Since no change in the sensitivity for CDDP was observed in all cells examined by treatment with Verapamil, it was confirmed that CDDP was not directly a substrate of Pgp. On the other hand, it was shown that Pgp was related to the sensitivity to DXR in each cell line because the sensitivity was enhanced 8.9-, 4.8-, 2.3- and 4.5-fold by the treatment of RPTEC, 14TKB, OS2 and RCW respectively. An improvement was seen in the level of IC50 of DXR, which was almost equal to or near to 0.4 μM without Pgp expression level in each cell. These results suggested that Pgp plays an important role in the resistance to DXR.

It is known that V-ATPase is the pump that maintains alkalinity in cytoplasm via the excretion of protons, thereby leading to resistance to CDDP and DXR by suppressing the intracellular accumulation of the basic drug (22). Expression of V-ATPase was not observed in OS2. In order to examine the relationship of V-ATPase to drug sensitivity, the effect of the V-ATPase inhibitor, Bafilomycin, on the cytotoxicity of anticancer drugs against each cell was measured. Although no significant increase in sensitivity to CDDP was observed by the treatment of RCW, OS2 and 14TKB with 1 μM Bafilomycin, the sensitivity to DXR in RCW and OS2 doubled, but there was no increase in 14TKB. However, the cell toxicity of Bafilomycin had no effect on the sensitivity in RPTEC. These results suggested that V-ATPase is not directly involved in drug resistance.

The expression level of CRR9, which selectively pumps CDDP, was lower in all tested RPTEC cells, and the sensitivity to CDDP in RPTEC was the same as in RCC. This finding suggested that CRR9 is not directly involved in drug resistance.

Although the expression level of CRR9, which is a selective excretion pump of CDDP, was high in all cells examined and highest in RPTEC, the sensitivity to CDDP in RPTEC was at the same level or higher than in RCC cells. This demonstrated a direct relationship between the expression level of CRR9 and CDDP resistance.

γGCS is a rate-limiting enzyme of GSH synthesis and intracellular GSH is maintained at an essentially high level. The expression level of γGCS was very low in OS2. A positive correlation between the γGCS expression level and each intracellular GSH content was observed; the GSH content was 22, 120, 4 and 25 nmol/mg protein in RPTEC, 14TKB, OS2 and RCW respectively (Table I). The degree of resistance to CDDP was high in RPTEC, 14TKB and RCW with a substantial intracellular GSH content. It was suggested that the cytocidal effect was suppressed by combining GSH with CDDP without the catalysis of GST because of nucleophilicity of GSH and nucleophilicity of CDDP (28). Treatment of RPTEC, 14TKB and RCW with BSO, an inhibitor of γGCS, decreased the intracellular GSH content to 4, 13 and 5 nmol/mg protein respectively. Lowering the GSH level by BSO-treatment increased CDDP sensitivity approximately 2- to 5-fold as the IC50 values of CDDP in RPTEC, 14TKB and RCW were 0.59, 1.5 and 1.5 μg/ml respectively. However, no significant effect of BSO on the DXR sensitivity was observed. This result was attributed to the disappearance of drug efficacy due to conjugation of GSH related to drug sensitivity (18-21), rather than to a reduction of superoxide and DNA repair through the protection effect of GSH.

The improved sensitivity to DXR, resulting from treatment of RCW, that led to high expression of MRP1 in BSO was an interesting finding. Although the mechanism of MRP1 (GS-X pump) function was assumed to be exclusion by the pump of GSH-conjugation to the drug and co-transportation of the drug and GSH, the mode of function of the transporting system remains unclear (3). The decrease in GSH content in 14TKB without MRP1 and RCW with MRP1, by treatment of BSO, enhanced the same degree of CDDP-sensitivity in both cells. However, the sensitivity to DXR was more enhanced in RCW than in 14TKB. These results suggested that detoxication of CDDP by the conjugation with GSH resulted in the resistance to CDDP and that, similarly, efflux of the DXR conjugated with GSH or co-transportation with GSH by the MRP1 pump led to resistance to DXR. The tolerance, by reduction of superoxide produced by treatment with these drugs, and repair of damaged DNA, through the protection effect of GSH, were more important for CDDP than for DXR. On the other hand, it has been reported that expression of both MDR1/Pgp and MRP2/cMOAT was induced in the

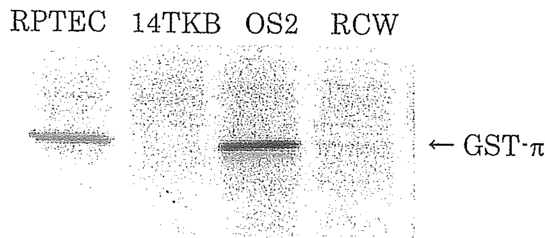

Figure 4. Expression of GST-π in RPTEC, 14TKB, OS2 and RCW cells by Western blot analysis using anti-GST-π antibody. Cell lysate extracted with 2% SDS was analyzed by SDS-PAGE on 12.5% polyacrylamide gel, and each band was transferred onto nitrocellulose filters.

rat kidney by administration of CDDP (50). It was suggested that these resistance factors might have induced high level expression via the treatment of RPTEC with CDDP.

GST activities in RPTEC, 14TKB, OS2 and RCW were 21, 7, 70, and 10 nmol/min/mg protein respectively, as shown in Table I. GST activity and GSH content were contradictory. On the other hand, the GST-π expression level correlated with GST activity, and the level in RPTEC and OS2 was high in all cells examined. However, the expression in 14TKB and RCW was not detectable (Fig. 4). GST-π (placental form GST isozyme), which is expressed at high levels in many cells undergoing carcinogenesis, has been reported to be a drug tolerance factor (28-31). We also reported that GST-π was a drug resistance factor because inhibition of GST activity and suppression of GST-π expression in rat hepatoma cells caused an increase in sensitivity to anticancer drugs (40,41). However, the expression of GST-π was observed in RPTEC, and the level of GST-π expression varied in several human RCC cell lines, including 14TKB, OS2 and RCW. Also, it was difficult to explain the drug sensitivity by the level of GST-π expression alone. Therefore, it was assumed that some drug resistance factors, such as efflux pumps of drugs rather than GST-π, affected the sensitivity (7)

A recent study revealed that a conjugate of DXR with GSH via glutaraldehyde (GSH-DXR), which shows rapid intracellular accumulation without efflux by Pgp, improved the cytotoxicity of DXR against MDR cells (40) and potently induced apoptosis in DXR-sensitive and -resistant cells relative to DXR (40). Moreover, we showed that GSH-DXR inhibited GST activity and suppressed GST-P (π) mRNA, indicating that inhibition of the enzyme makes an important contribution to the manifestation of potent cytotoxicity of GSH-DXR against rat hepatoma AH66 cells (50- to 100-fold compared with that of DXR) (41,42). Recent reports demonstrated that the elevation of human GST-π gene expression and enzyme activity was associated partly with MDR (28-31). GSH-DXR exhibited potent cytotoxicity in comparison with DXR for RPTEC and RCC cell lines and the IC50 values of GSH-DXR were 5.5, 11.0, 10.0 and 1.8 nM in RPTEC, 14TKB, OS2 and RCW respectively. It was suggested that this drug reversed the effects of various resistance factors such as Pgp, MRP and GST-π.

The characters (degree of resistance) of RCC cell lines vary in the expression level of many resistance factors in comparison with normal cells. Therefore, these findings suggest that medical treatment should be conducted after investigating the expression of each resistance factor, at least in chemotherapy. On the other hand, the important finding that GSH-DXR suppressed GST-π with the target, and also reversed resistance factors such as Pgp and MRP, suggests that GSH-DXR may be an effective reverser of renal cell carcinomas that express the transporter of ions and various molecules.

Further study will attempt to identify whether or not GSH-DXR is a specific and effective reverser for RCC cell lines.

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