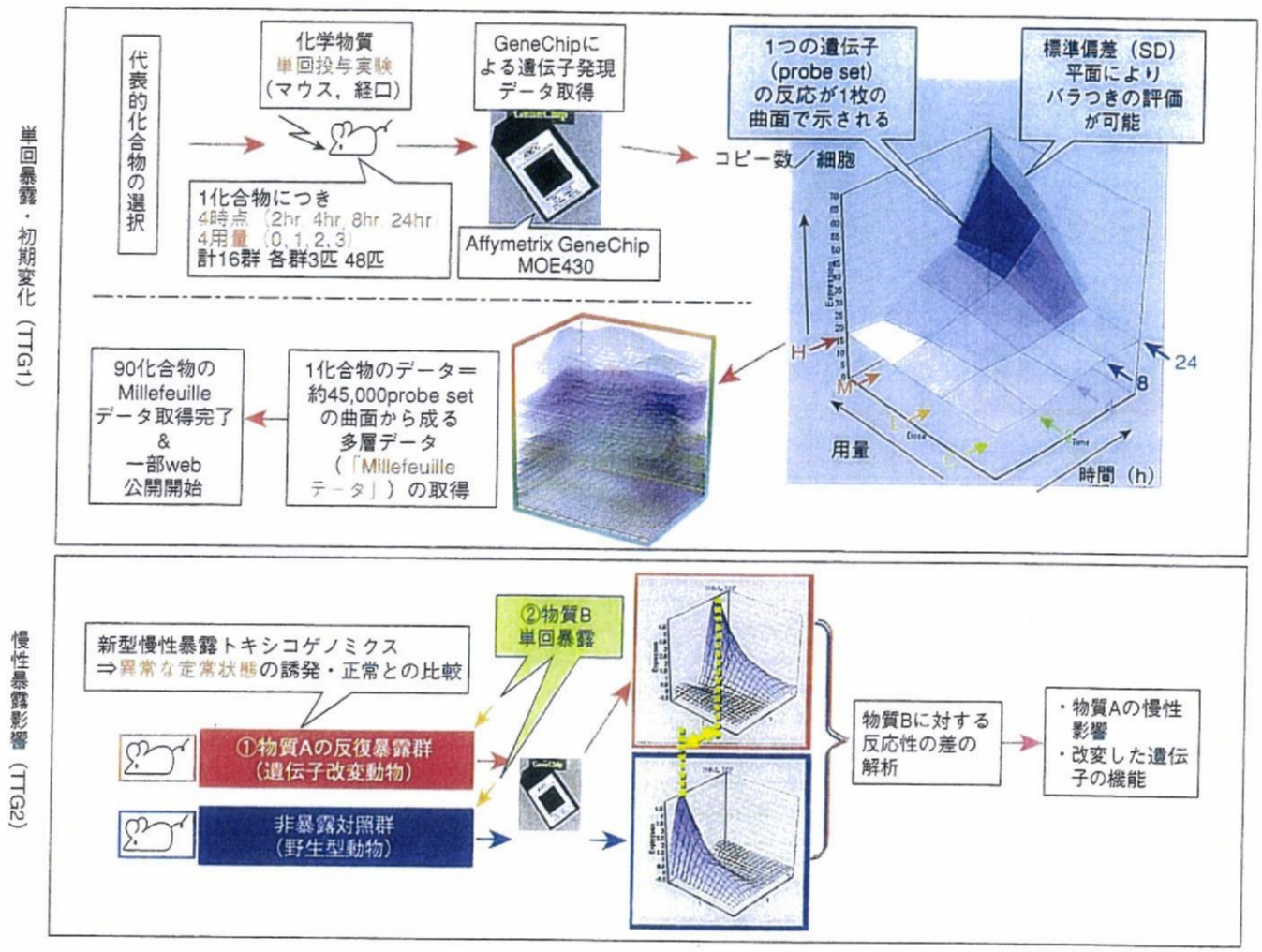

図2. Percellome法と3次元表示による多層（Millefeuille）データシステムを用いたプロジェクトの根幹部分の概要
単回投与による遺伝子発現初期変化データを90化合物について取得（上段）．現在，反復投与の影響を検討中（下段）．H；高用量（high），M；中用量（medium），L；低用量（low），C；コントロール（control）．

て，Gene Ontologyなどの既存知識を利用して候補遺伝子リストの理解を支援するソフトウエア（MF GoPlot）を用意した．このツールは一種の化合物クラスタリングとしても利用することができる．

さらに候補遺伝子リストを基に複数化合物間比較を行い，複数条件下においても同期して発現する遺伝子群を自動抽出するシステムも開発済みである．本システムで得られた同期遺伝子群はシグナルカスケードの構成単位である可能性があり，データベース化しつつ，その解析を進めている（5TB規模のデータベース部分および，大量計算アルゴリズム実装は（株）NTTコムウェアおよび（株）日本NCR/Teradataとの共同開発による）．

Ⅲ．Percellome手法のリアルタイムPCRを含む他のプラットホームへの適用

Percellome手法は，GSCの受け入れ条件を整えることにより，様々なプラットホームに適用可能である．その1つとして最も定量性が高いとされるリアルタイムPCR（ABI PRISM 7900 HT・96ウェルプレート）への適用例を示す．現行のRT-PCR絶対定量法では，遺伝子ごとに検量線が必要であり，多数のサンプルについて多数の遺伝子を検討するには不向きである．Percellome RT-PCRでは，マイクロアレイと同様の原理を用いる．すなわち，サンプル破砕液に，その細胞数に比例する量のスパイクカクテル（GSC）を添加し，それらのCt値をPCRプレートごとの検量線とすることにより，測定したい遺伝子のCt値を細胞1個当たりのmRNAコピー数に換算する．これにより，GAPDHやActinなどのハウスキーピング遺伝子が変動してしまう際の問題，例えば，少数の遺伝子を検討する際にGlobal normalization法を適用し難い問題などが解決される．共通サンプルを測定しデータを比較することにより，Affymetrix GeneChipのPercellome結果と9割程度の整合性が確認され，

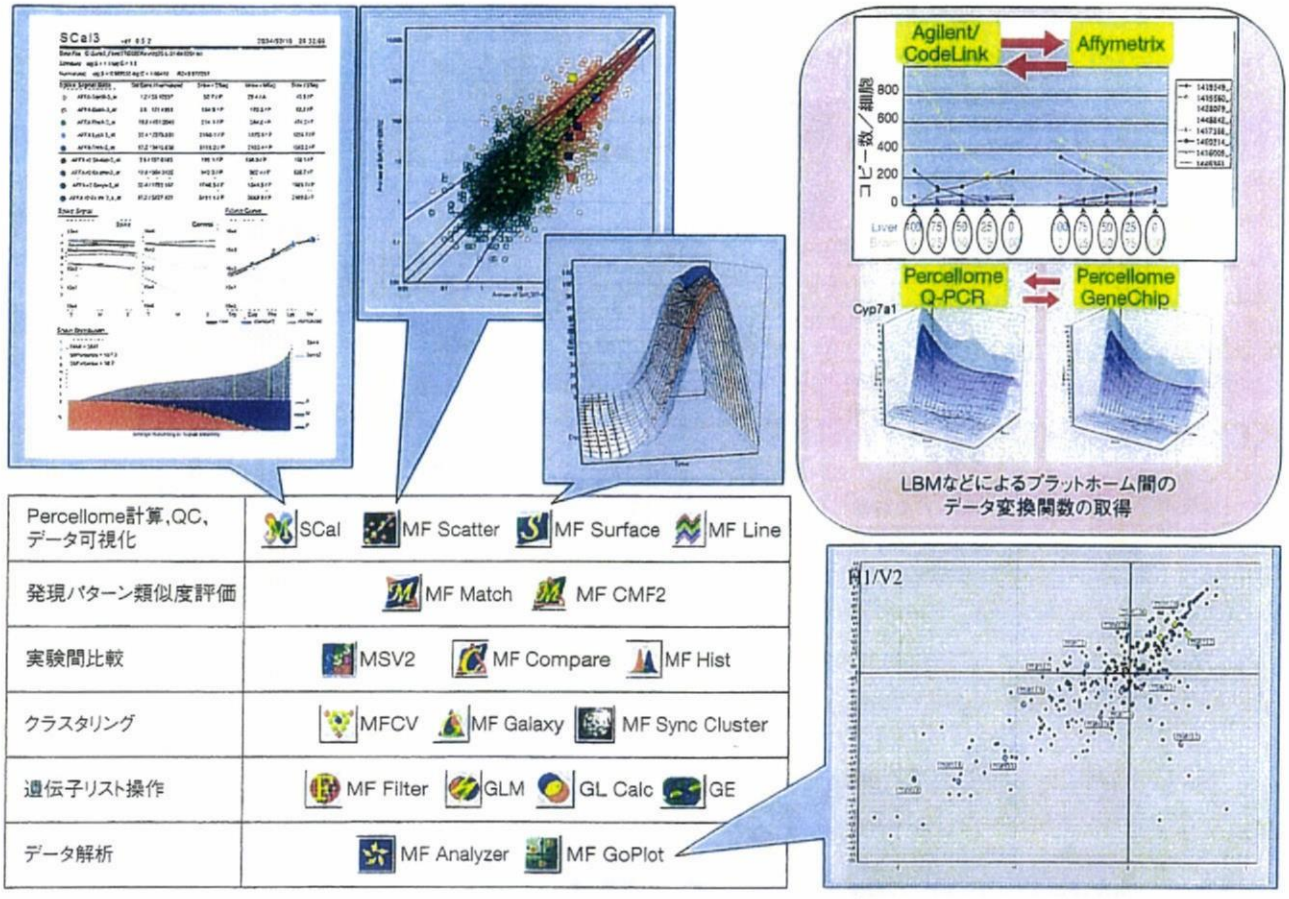

図3．3次元多層（Millefeuille）データの解析などに用いる独自開発プログラム群

品質管理とともにPrecellome計算を自動的に実施するSCal，Plotソフトウエア，3次元曲面の描画ソフト（MF Surface），など．右上はプラットホーム間のデータ変換情報の得方を示す．LBMを用いる方法（上段）と，実際の実験サンプルを用いる方法（下段）がある．いずれも，一度，両方のプラットホームでそれらのサンプルを測定する必要がある．

GeneChipとPercellome RT-PCRとの間でのコピー数の換算式がいくつかの遺伝子について得られている．この他に，Agilent社製の単色マイクロアレイとCodeLinkアレイにGSCを測定可能なカスタムアレイを用意し終え，LBMサンプルのデータなどをもとに，これらとの間の換算式も得つつある（図3右上）．

Percellome法は，Affymetrixの新しいエキソンアレイの定量性・直線性の検討にも適応可能である．Affymetrix社のHuman Exon 1.0 ST Arrayと従来型の発現アレイHuman Genome U133 plus 2について，性質の異なるヒト癌細胞株2株から調製したLBM様標準サンプル（100：0，75：25，50：50，25：75および0：100混合5サンプル）による比較を行い，両アレイ間の相関性の高いprobe setを多数検出することができた．また，既知のエキソンに対して設計されたprobe setでは発現が見られ，イントロンに対して設計されたprobe setでは発現が見られない，あるいは，既知のsplicing variantに対応したprobe setの発現が検出された，などの基本性能が確認された．しかし，Percellome法を適用して未知のsplicing variantの検出力を向上させるためには，現状では各エキソン間の定量性に問題があることが示唆された．定量値を算出する補正アルゴリズムの開発など，何らかの対策が必要であることが考えられ，現在，Affymetrix社に確認を行っている．

Ⅳ．核内受容体原性毒性のPercellomeトキシコゲノミクス解析

受容体原性毒性とは，化学物質が受容体（リガンド依存的転写因子を含む）に選択的に結合してシグナルをかく乱し，その結果生じる有害性を指す．代表例としてはダイオキシンが挙げられる．AhR（Arylhydrocarbon receptor）ノックアウトマウスでは，ダイオキシンを大量に投与しても毒性がほとんど観察されない．すなわち，野生型マウスがダイオキシンで死ぬメカニズムには，AhRが必須であり，AhRからの異常なシグナルがマウスを死に至らせていることに

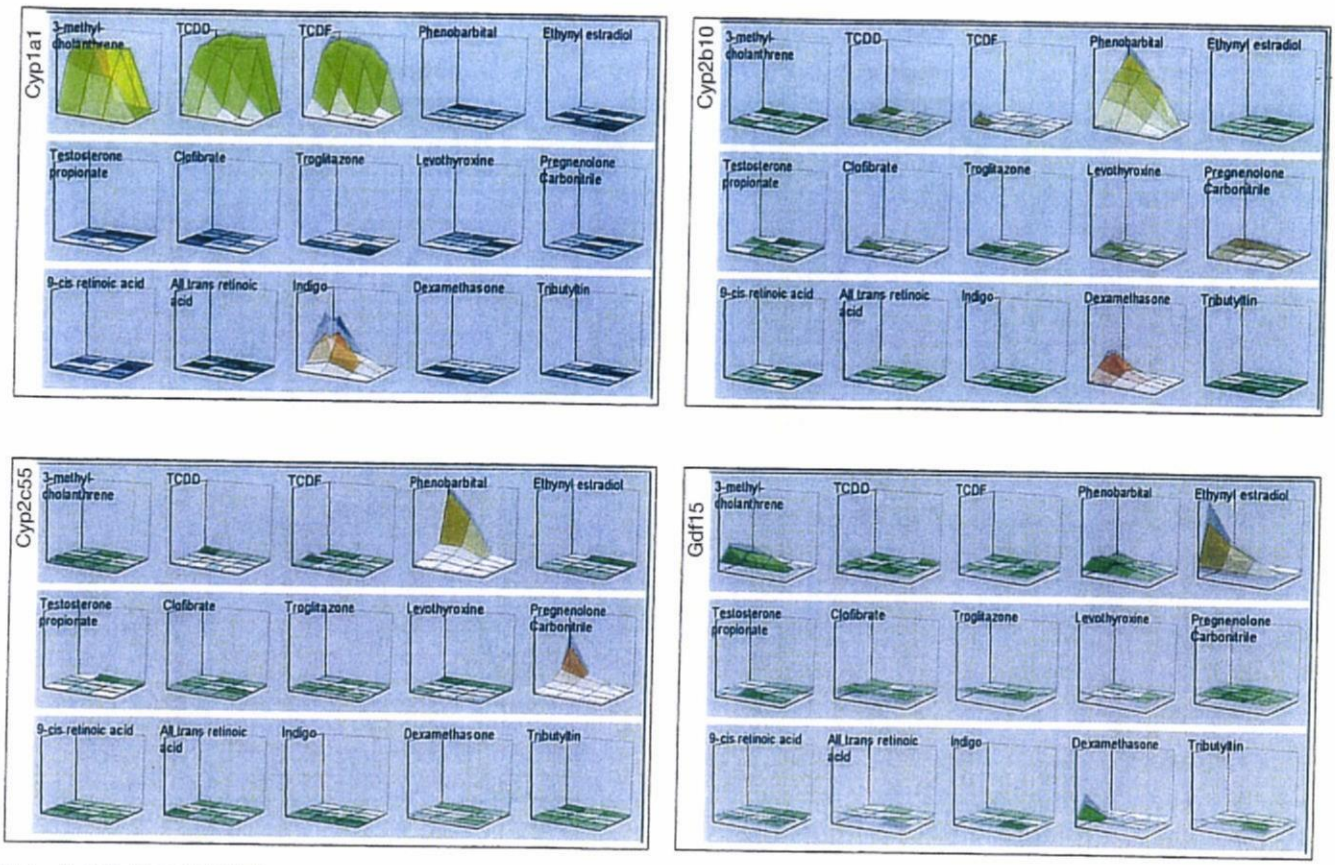

図4. 化合物間の発現比較

15種類の核内受容体リガンド化合物（各3次元グラフ内に表示）によるCyp1a1（左上）、Cyp2c55（左下）、Cyp2b10（右上）および、Gdf15（右下）の遺伝子発現を3次元表示したもの．各軸は，図2のとおり．縦軸のスケールは遺伝子ごとに共通．リガンドに選択的な遺伝子の発現が確認される．

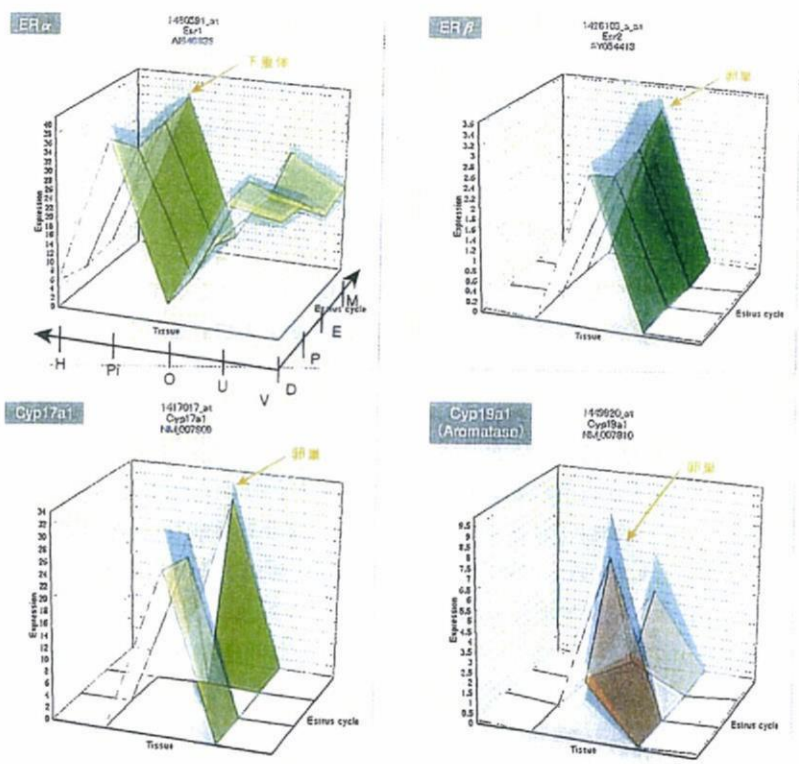

図5. 臓器間の発現比較

マウスの性周期（Diestrus、Proestrus、Estrus、Metestrusの4日間で1周期）ごとの視床下部（H）、下垂体（Pi）、卵巣（O）、子宮（U）および膣（V）における、ERα、ERβ、Cyp17a1（steroid-17α-hydroxylase）、およびCyp19a1（Aromatase）の遺伝子発現変動を3次元表示したもの．後二者の酵素は卵巣において周期性を持って発現している．

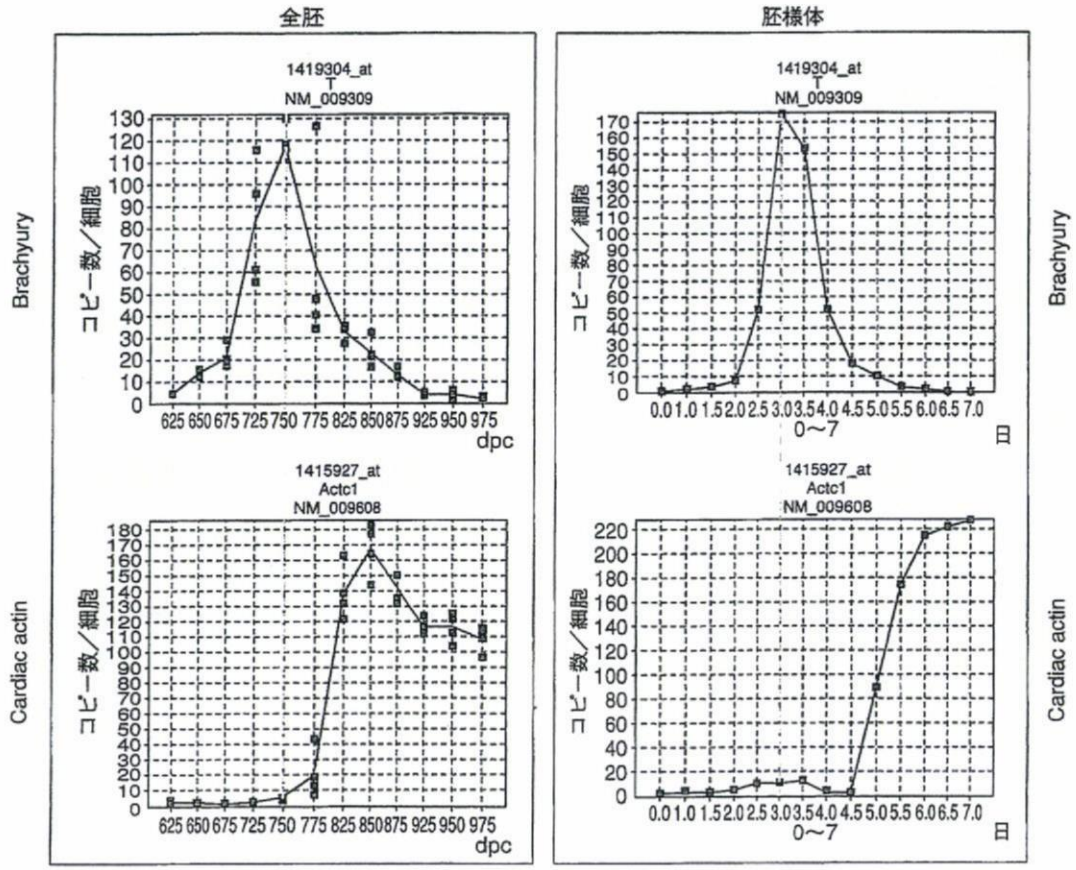

図6. マウス胎児（全胚）と胚様体の発現比較

マウス全胚の胎生6.25日〜9.75日までの遺伝子発現と，胚様体の1日〜7日目までの遺伝子変動の網羅的データベースから，初期中胚葉分化マーカーであるBrachyury遺伝子と，Cardiac actin遺伝子の経時変化を示す．

なる．エストロジェン活性化学物質による有害影響（内分泌かく乱化学物質問題）も同様にER（estrogen receptor）を介する受容体原性毒性と考えられ，胎生期にERを発現する組織が，低用量シグナルかく乱影響の重要標的であると考えられている．

ここでは，受容体原性毒性研究の基盤として，Percellome手法を適用して，①核内受容体作動性物質によるマウス雄肝臓の遺伝子発現変動，②性周期に伴うマウス雌生殖器遺伝子発現変動，③生後の発達過程におけるマウス雌生殖器遺伝子発現変動，の3種類のデータベースを構築した．例えば，①では10種類の核内受容体に作用する典型物質について，単回経口投与後，2，4，8，24時間目の変動を解析し，Ethynyl-estradiolがGDF15，TCDDがCyp1a1，9-cis Retinoic Acidが Cyp26a1，Dexamethasoneが Cyp2b10，Clofibrateが Cyp4a14，PCNが Cyp2c55など，各々の受容体に特徴的な遺伝子発現を誘導するところがとらえられている（図4）．②の性周期データベースは視床下部，下垂体，卵巣，子宮，膣を対象としており，性周期との関連が網羅的にとらえられている（図5）．これらのデータベースは，今後，各種の候補物質が引き起こす変化を詳細に解析する際の基準として利用される．

V．発生トキシコゲノミクスへの応用

発生毒性学は，個体発生過程におけるダイナミックな遺伝子発現調節の分子機構を把握することにより，さらに正確なものに補強されると考える．現在，C57BL/6マウス胚の器官形成期初期にあたる胎生6.5〜9.5日（プラグ確認日：0.5日）の，①全胚の遺伝子発現変動解析，②遺伝子欠失マウス全胚との比較，および③標的が明らかな既知発生毒性物質投与による本データベースの具体的な適用，を実施している．①についてはすでに0.25日間隔（Time point 計12点）の遺伝子発現データベースを得て，②遺伝子欠失胚のデータといくつかの注目すべき遺伝子についてはwhole mount ISHを用いた発現の検証を加えた．これと並行して，ES細胞からhanging drop法で得た胚様体の0.5日間隔の遺伝子発現データとの比較を実施している．個体発生に関与

する遺伝子群の多くは経時的に激しく変化しており，既知発生毒性物質投与実験については標的遺伝子シグナルカスケードを解析中である（図6）.

おわりに

ノーザンブロットでは実験サンプルにだけバンドが見られ，対照サンプルには遺伝子発現がないという結果を得ても，細胞1個当たりで定量してみると，対照が10コピーに対して実験サンプルが20コピーである場合がある．"無"が"有"になったのではなく，"10"が"20"になったのである．

さて，筆者らの属する毒性学でも，医学の分野でも，疾患概念や毒性概念が整理され，患者や実験動物を診断する際には，まず，そのどれに当てはまるかを検討する．すなわち，どの"典型"に近い症例であるかを検討することから始まることが多い．

しかし，最近の医学・生物学には多因子疾患・多因子形質発現制御の概念が導入され，今から何年かの後には，"21世紀初頭までは，患者の遺伝子多型を調べずして治療を行っていた時代"として，"血液型を調べずに輸血していた時代"と並び称されるようになる可能性がある．このような多因子概念が定着すると，その多くは，"有（100%）""無（0%）"の組み合わせではなく，"70%""50%""90%"といった半端な数の組み合わせであることが考えられる．すなわち，今までの離散値的な"典型"例を基準とするアプローチから，連続値的な病態"スペクトラム"を直接扱うアプローチに変革していく可能性が考えられる．その際の網羅的データの解析とその蓄積の必要性を考えるとき，遺伝子発現データの定量化・標準化という問題は，今まで以上に重みを増すと考えられる．生命現象の網羅的解析にはトランスクリプトームだけでは不十分であることは自明であるが，この定量性を確保することは，これから実現されるであろう網羅的プロテオミクスなどの基盤としても重要ではないかと考える．

マイクロアレイなどから得られるトランスクリプトーム情報が，今後の医薬品審査や化学物質の安全性評価の際に必須なものとなる時代がすぐそこまで来ていることを念頭に，筆者らはPercellome法をさらに展開し，Percellome Projectデータベースを可能な限り高精度に保ちつつ毒性学的な内容を充実させるべく最大限の活動を継続して行く所存であるが，この技術，あるいは研究内容が毒性学以外の研究分野にもお役に立つことができれば幸甚である．

謝辞　本システムの開発とプロジェクトの遂行に当たっては，当毒性部の全メンバー，特に松田菜恵，辻 昌貴，森田紘一，今井あや子，安東朋子，安部麻紀，森山紀子，近藤優子，青柳千百合，相原妃佐子，渡辺 忍の各氏の卓越した働きに深謝する．本研究は厚生労働科学研究費補助金H13-生活-012，H13-生活-013，H14-トキシコ-001，H15-化学-002，H18-化学-一般-001などによる．

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A POSSIBLE MECHANISM FOR THE DECREASE IN SERUM THYROXINE LEVEL BY POLYCHLORINATED BIPHENYLS IN WISTAR AND GUNN RATS

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Introduction

Most polychlorinated biphenyl (PCB) congeners are known to decrease the levels of serum thyroid hormone and to increase the activities of hepatic drug-metabolizing enzymes in rats [1,2]. As possible mechanisms for the PCB-mediated decrease in level of serum thyroid hormone, enhancement of thyroid hormone metabolism by PCBs and displacement of the hormone from serum transport proteins (transthyretin (TTR)) are considered [3-5]. Especially, the decrease in the level of serum thyroxine (T_4) by 3,3',4,4',5-pentachlorobiphenyl, Aroclor 1254, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in rats is thought to occur mainly through the induction of the UDP-glucuronosyltransferase (T_4-UDP-GT) responsible for glucuronidation of T_4 [2,4]. However, the magnitude of decrease in level of serum total T_4 is not necessarily correlated with that of increase in T_4-UDP-GT activity [1,6]. Recently, we suggested that the decrease in serum total T_4 level by a single administration of either Kanechlor-500 (KC500) or 2,2',4,5,5'-pentachlorobiphenyl in UGT1A-deficient Wistar rats (Gunn rats) was not dependent on the increase in hepatic T_4-UDP-GT activity, and further suggested that even in Wistar rats, the PCB-mediated decrease in serum T_4 level might occur not only through the increase in hepatic T_4-UDP-GT [7]. In the environment, humans and animals are exposed to PCB of very low level extent over a long period of time.

In the present study, therefore, to clarify possible mechanisms for the PCB-mediated decrease in level of serum thyroid hormone, we examined a relationship between the decrease in serum total T_4 level and the increase in the hepatic T_4-UDP-GT (UGT1A1 and UGT1A6) by the consecutive treatment of Wistar and Gunn rats with PCB and demonstrated that the PCB-mediated decrease in serum total T_4 level in rats was not necessarily dependent on the increase in hepatic T_4-glucuronidation, but the decrease occurs through the increased transport of T_4 to the liver.

Materials and Methods

Animal treatments. Male Wistar rats (160-200 g) and Gunn rats, (180-210 g) were obtained from Japan SLC., Inc. (Shizuoka, Japan). Male Wistar and Gunn rats were housed in three or four per cage with free access to commercial chow and tap water, and were maintained on a 12-h dark/light cycle (8:00 a.m.-8:00 p.m. light) in an air-controlled room (temperature: 24.5 ± 1℃, humidity: 55 ± 5%), and were handled with humane care under the guidelines of the University of Shizuoka (Shizuoka, Japan). The rats were treated with ip injection of KC500 (10 mg/kg) dissolved in Panacete 810 (5 ml/kg) at 24 h-intervals for 10 days. Control animals were treated with vehicle alone (5 ml/kg).

A) *In vivo* study. Rats were killed by decapitation on day 4 after the final dosing, and the liver was removed, and hepatic microsomes were prepared according to the method of Kato *et al.*[8] and stored at –85℃ until used. Blood was collected from each animal between 10:30 and 11:30 a.m. After clotting at room

temperature, serum was separated by centrifugation and stored at −50℃ until used.

Analysis of serum hormones. The levels of total T_4, free T_4, and thyroid-stimulating hormone (TSH) were measured by radioimmunoassay using the T4-RIABEAD (DAINABOT Co., Ltd, Tokyo, Japan), free T_4 (Diagnostic Products Corporation; Los Angels, CA), and Biotrak rTSH [^{125}I] assay systems (Amersham Life Science Ltd.; Little Chalfont, UK), respectivery.

Hepatic microsomal UDP-GT assay. The amount of protein was determined by the method of Lowry *et al.*[9] with bovine serum albumin as a standard. The activity of microsomal UDP-GT toward T_4 was determined by the method of Barter and Klaassen [3]. The UDP-GT activity was measured after activation of the UDP-GTs by 0.05% Brij 58.

Western blot analysis. Polyclonal anti-peptide antibodies against the common region of UGT1A isoforms and specific antibodies against UGT1A1, UGT1A6, and UGT2B1 were used [10]. Western analyses for microsomal UGT isoforms were performed by the method of Luquita *et al.*[11]

B) *Ex vivo* study. At 4 day after last treatment with KC500, animals were anesthetized with 50 mg/ml sodium pentobarbital combined 1:1 with 1 mg/ml potassium iodide at 2 mg/ml. The femoral artery was cannulated and primed with heparinized saline. Fifteen minutes later, animals were given [^{125}I]T_4 i.v. at 15 μCi/ml in 10 mM NaOH saline including 1 % normal each animal serum.

Clearance of [^{125}I]T_4 from serum. Five minutes following i.v. administration of [^{125}I]T_4 and five more times at the indicated time, a portion of blood was sampled from the artery, and serum was collected and stored at -50℃ for assay. Two aliquots were taken from serum samples for γ-counting.

Analysis of [^{125}I]T_4 binding to serum proteins. The levels of serum [^{125}I]T_4-albumin, [^{125}I]T_4-thyroxine binding protein, and [^{125}I]T_4-TTR complexes were determined by the method of Kato *et al.*[12]

Tissue distribution of [^{125}I]T_4. At 60 min after the administration of [^{125}I]T_4, blood was sampled from abdominal aorta, and tissues were removed and weighted. Radioactivities in serum and tissues were determined by γ-counting, and concentration ratios of the tissue to serum were determined.

Results and Discussion

The serum total T_4 and free T_4 levels were markedly decreased not only in the Wistar rats but also in the Gunn rats 4 days after final treatment with KC500 (10 mg/kg, i.p., once daily for 10 days), and there was no significant difference in magnitude of the decrease between Wistar and Gunn rats (Fig. 1). At the same time, the level and activity of T_4-UDP-GT (UGT1A1 and UGT1A6) were significantly increased by treatment with KC500 in Wistar rats but not in Gunn rats (Fig. 2). In contrast, the level of UGT2B1 was increased by KC500 in both Wistar and Gunn rats. Hepatic microsomal enzyme activities (benzyloxyresorufin *O*-dealkylase activity, 48- and 35-fold; pentoxyresorufin *O*-dealkylase activity (CYP2B1/2), 17- and 10-fold; ethoxyresorufin *O*-dealkylase activity (CYP1A1/2), 64- and 10-fold in Wistar and Gunn rats, respectively) were significantly increased by KC500 treatment. In addition, no significant change in the level of serum TSH by the KC500 treatment was observed in either Wistar or Gunn rats.

Furthermore, significant increases in the disappearance of [^{125}I]T_4 from the serum and in the distribution volume of [^{125}I]T_4 by KC500 treatment were observed in both Wistar and Gunn rats. A concentration ratio of the liver to serum was approximately one in either Wistar or Gunn rats, and KC500-treatment increased the ratio by 4 times. The concentration of [^{125}I]T_4 appeared to be the highest in the liver in both Wistar and Gunn rats. The hepatic levels of [^{125}I]T_4 in both rats were further increased by KC500 treatment. More than 40% of [^{125}I]T_4 dosed was transported to the liver of both rats (Fig. 3). In contrast, a significant increase in liver weight was observed in KC500-treated Wistar rats but not in the Gunn rats. In addition, significant decrease in the binding of [^{125}I]T_4 to serum TTR and significant increase in the binding to serum albumin by KC500 treatment were observed in either Wistar or Gunn rats.

In conclusion, the present findings demonstrate that the PCB-mediated decrease in serum total T_4 level in Gunn rats occurs without an increase in hepatic T_4-UDP-GT activity; they further suggest that in both strain rats, the PCB-mediated decrease occurs through the increased transportation of T_4 to the liver. Furthermore, the decrease in the binding of T_4 to serum TTR and hepatic hyperplasia might be attributed to the increase in the level of T_4 in the liver. In Wistar rats, however, the PCB-mediated induction of T_4-UDP-GT might, at least in part, contribute to the decrease. Further studies are necessary for understanding the susceptibility toward a PCB-mediated decrease in serum T_4 level in animals including humans.

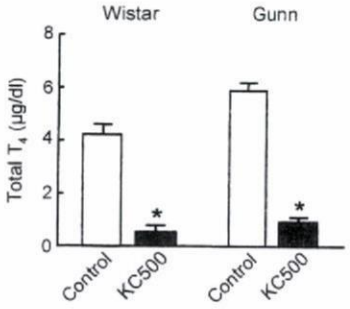

Fig. 1. Effect of KC500 on the level of serum total thyroxine in Wistar and Gunn rats

Animals were killed 4 days after the final administration of KC500 (10 mg/kg, i.p., once daily for 10 days). Each column represents the mean ± S.E. (vertical bars) for five to six animals. *P<0.01, significantly different from each control.

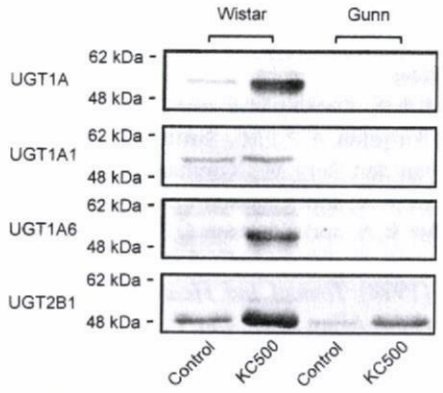

Fig. 2. Representive Western blot patterns for hepatic microsomal UGT isoforms in KC500-treated Wistar and Gunn rats

Animals were killed 4 days after the final administration of KC500 (10 mg/kg, i.p., once daily for 10 days).

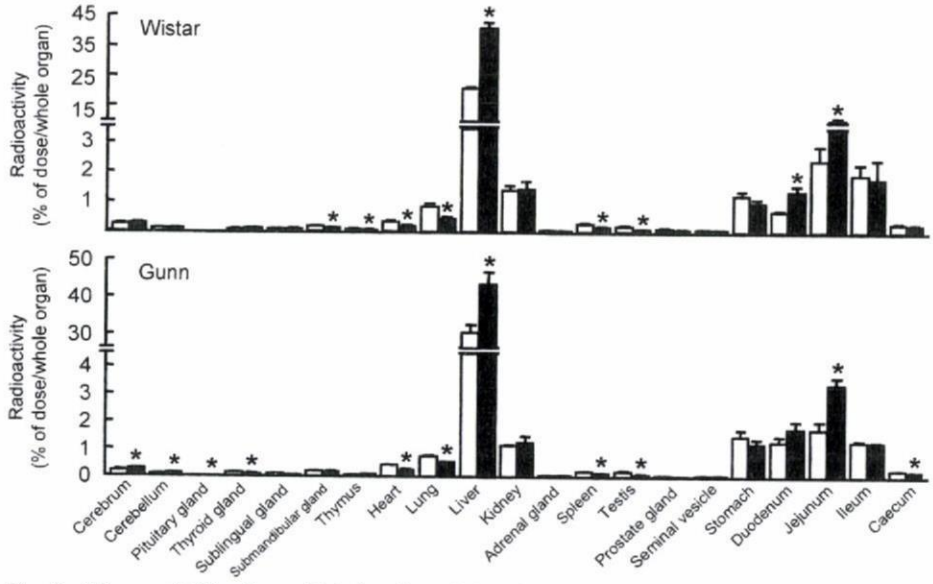

Fig. 3. Tissue distribution of total radioactivity after the administration of [^{125}I]T_4 to the KC500-treated Wistar and Gunn rats

KC500 (10 mg/kg) was given i.p. to animals at 24 hr-intervals for 10 days. The radioactivity of each tissue was measured at 60 min after the i.v. administration of [^{125}I]T_4. Each column represents the mean ± S.E. (vertical bars) for three to six animals. *P<0.05, significantly different from each control. □, control; ■, KC500.

Acknowledgements

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Original Paper

PTOV1: a novel testosterone-induced atherogenic gene in human aorta

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Abstract

There are gender differences in the development of atherosclerosis, possibly owing to differences in sex steroid hormone action and/or metabolism. One of the atherogenic effects of testosterone is thought to be androgen receptor (AR)-mediated vascular smooth muscle cell (VSMC) proliferation. However, the detailed mechanism of this effect, particularly the identity of the genes associated with VSMC proliferation, remains largely unknown. Therefore, we first employed microarray analysis and, subsequently, quantitative RT-PCR to analyse RNA expression in AR-positive human VSMCs treated with testosterone in order to detect testosterone-induced genes associated with cell proliferation. We further examined whether the genes identified were involved in cell proliferation using small interfering RNA (siRNA) transfection. Expression of the gene products was then evaluated in human aorta with various degrees of atherosclerosis in order to evaluate the clinical relevance of the findings. Both microarray and quantitative RT-PCR analyses demonstrated marked induction of the human prostate overexpressed protein 1 (*PTOV1*) gene by testosterone in the cell lines: this gene was recently identified as a novel androgen-induced gene involved in prostate tumour cell proliferation. Inhibition of *PTOV1* by transfection of its corresponding siRNA suppressed testosterone-induced cell proliferation. In human aorta, PTOV1 immunoreactivity in the nuclei of neointimal VSMCs was abundantly detected in male aorta with mild atherosclerotic changes compared with female aorta or male aorta with severe atherosclerotic changes. These findings indicate that the *PTOV1* gene is androgen-responsive in VSMCs and that it may play an important role in androgen-related atherogenesis in the human aorta, particularly early atherosclerosis in the male aorta, through regulating proliferation of neointimal VSMCs.

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Keywords: vascular smooth muscle cells; androgen receptor; testosterone; cell proliferation; PTOV1

Introduction

There is an important, well-documented, gender difference in coronary heart disease risks, with earlier onset of disease and excess mortality in male subjects [1–3]. Athero-protective effects of oestrogens on vascular structure and function have been proposed as one of the most important mechanisms accounting for this gender difference [4]. On the other hand, an association between androgens and atherosclerosis continues to be disputed. Androgens have been considered to reduce the incidence of ischaemic myocardial disease in men, but they have also been reported to exert atherogenic effects on the human cardiovascular system through promoting plaque formation and enhancing monocyte adhesion to endothelial cells [5–8]. It has been demonstrated that testosterone exerts direct atherogenic effects by promoting cell proliferation through an initial interaction with the androgen receptor (AR) in vascular smooth muscle cells (VSMCs) *in vitro* [9]. However, unlike oestrogens, the possible effects of testosterone on atherogenesis and/or anti-atherogenesis have not been extensively studied. It is therefore important to study the detailed mechanisms of these direct effects of testosterone on the human cardiovascular system.

In this study, we first screened for testosterone-induced genes involved in the proliferation of VSMCs using microarray analysis in cell lines derived from AR-positive human VSMCs. We then confirmed the results by employing other *in vitro* studies. As testosterone induced marked overexpression of *PTOV1* in

these assays, we subsequently examined the levels of expression of PTOV1 protein in VSMCs in samples of the human abdominal aorta obtained at autopsy.

Materials and methods

Vascular smooth muscle cells

Two types of human dedifferentiated VSMCs, ie HUVS-112D (derived from human umbilical cord), and T/G HA-VSMC (derived from human aorta) were commercially obtained from American Type Culture Collection (Manassas, VA, USA) [10,11]. We examined whether these cells expressed AR using an RT/real-time PCR with a light Cycler System using DNA binding dye SYBR Green I, and immunoblotting with AR polyclonal antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA), as reported previously [10,12].

GeneChip microarray assay

The VSMCs above were cultured until a sub-confluent state was achieved. The medium was then replaced with fetal bovine serum (FBS)-free and phenol red-free medium to arrest cell proliferation. After 24 h, the medium was replaced again with phenol red-free and FBS-free medium in the presence of testosterone (10 nM) or vehicle (0.1% ethanol). After incubation for 2 h, the cells were subsequently subjected to total RNA extraction for microarray analysis. Isolated total RNA was labelled as described in the Affymetrix (Santa Clara, CA, USA) GeneChip Expression Analysis Technical Manual (revision 3), as previously described [10]. The ratios represent the values up- or down-regulated by 10 nM testosterone treatment compared with control. We independently repeated the same experiment twice. Genes for which the average ratios increased more than 1.5-fold in both experiments using 10 nM testosterone treatment were considered up-regulated via AR when compared with control values [13]. When studying the potential functions of these genes, we used the homepage of the HUGO Gene Nomenclature Committee (http://www.gene.ucl.ac.uk/nomenclature, accessed 28 March 2006) for further examination of whether any had been previously reported to be involved in cell proliferation and to be associated with androgen effects. In this study, among the genes that were found to be significantly induced by testosterone treatment by microarray analysis, we regarded a gene that was up-regulated, and was known to be associated with both cell proliferation and androgenic effects, as a target gene.

Quantitative real-time PCR

After achieving sub-confluence and following growth arrest states of the VSMCs as described above, the medium was replaced again with phenol red-free and FBS-free F12-K medium with testosterone (10 nM), testosterone (10 nM) with flutamide, an AR-blocker (100 nM), or vehicle. After incubation for 2 h, the cells were subsequently subjected to total RNA extraction for RT/real-time PCR analysis, described previously [10]. mRNA levels for the target gene *PTOV1* were determined in each VSMC as a ratio relative to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and evaluated as a ratio (%) compared with that of each control cDNA. The analyses with real-time PCR were repeated three times. Table 1 summarizes the primers used [14].

siRNA preparation, transfection, and cell count assay

Small interfering RNAs (siRNAs) corresponding to *PTOV1* (Table 2) were synthesized based on results of a previous report, and were transfected into the VSMCs [15]. These VSMCs were seeded in a 24-well plate at an initial concentration of 50 000 cells/well with F-12K medium containing 2% FBS and were cultured until a sub-confluent status was achieved. The medium was then replaced with phenol red-free and FBS-free medium to arrest cell proliferation. After 24 h, transfection experiments of siRNA for endogenous gene targeting (10 nM or 100 nM) were carried out using RNAiFect™ transfection reagent (Qiagen, Valencia, CA, USA). After transfection, the cells were incubated in phenol red-free medium containing 2% dextran-coated, charcoal-stripped FBS with testosterone (10 nM) or vehicle (0.1% ethanol) for 24 h. We measured the number of cells in each sample as described above with Cell Counting Kit-8 system (Wako, Tokyo, Japan) after incubation for 48 h. We also examined the number of cells treated with

Table 1. Primer sequences used in RT-PCR analysis

cDNA	Sequence	Size (bp)
AR	Forward 5′-CTCACCAAGCTCCTGGACTC-3′ Reverse 5′-CAGGCAGAAGACATCTGAAG-3′	246
GAPDH	Forward 5′-TGAACGGGAAGCTCACTGG-3′ Reverse 5′-TCCACCACCCTGTTGCTGTA-3′	307
PTOV1	Forward 5′-CACCATCCCTCCATGTTGCTG-3′ Reverse 5′-TCTTCATTGGCCTCATCCCC-3′	250

Table 2. Sequences used in siRNA transfection analysis

cDNA	Sequence
PTOV1	Sense r(CAACAAGUUUCUGGCAUGG)dTdT Antisense r(CCAUGCCAGAAACUUGUUG)dTdT
Negative control	Sense r(UUCUCCGAACGUGUCACGU)dTdT Antisense r(ACGUGACACGUUCGGAGAA)dTdT

The target gene in this study (*PTOV1*) was determined by microarray analysis. The sequences of *PTOV1* siRNAs are based on a previous report [15].

transfection of negative control siRNA with scrambled sequences (Table 2), and treated with testosterone (10 nM) or vehicle. In order to evaluate transfection efficiency, we examined relative *PTOV1* mRNAs levels in these cells at 24 h after transfection of the specific siRNAs. The mRNA levels in each VSMC were calculated as a ratio relative to *GAPDH*, and were normalized to the ratio after transfection of negative control siRNA (10 nM).

Quantitative RT-PCR analysis of *PTOV1* mRNA expression in human aorta

Samples of human abdominal aorta were collected at autopsy from patients without a history of hormone replacement therapy. Autopsies were performed on 32 subjects (16 male, 16 female; mean 60.7 ± 3.3 years) in Tohoku University Hospital (Sendai, Japan) within 2 h post mortem. The Ethics Committee at Tohoku University School of Medicine approved the research protocol for this study. Aortic specimens were tentatively classified into the following four groups according to the sex of the deceased patient and degree of atherosclerosis, as previously described: group A = male, mild atherosclerosis, corresponding to groups I–III in the American Heart Association (AHA) classification; group B = male, advanced atherosclerosis, corresponding to groups IV–VI in the AHA classification; group C = female, mild atherosclerosis; and group D = female, advanced atherosclerosis) [10,11]. The distribution of the cases among these groups was as follows: A, 8 cases (mean 44.3 ± 10.6 years); B, 8 cases (mean 71.3 ± 3.7 years); C, 8 cases (mean 52.0 ± 3.9 years); and D, 8 cases (mean 75.0 ± 2.1 years), respectively. For RT/real-time PCR analysis, these specimens were treated according to our previous report [10]. The mRNA levels for *PTOV1* and *AR* in each sample are given as a ratio relative to *GAPDH*, and evaluated as a ratio (%) compared with that of each control cDNA.

Immunohistochemical analysis for PTOV1 protein expression in human aorta

Details of immunohistochemical procedures have been previously described [10,11]. We used immunostaining with diaminobenzidine (DAB) for immunohistochemical analysis of PTOV1 protein (using a monoclonal anti-human PTOV1 antibody; Novocastra Laboratories, Newcastle, UK) and AR (using a monoclonal antibody for human AR; Dako Corporation, Carpinteria, CA, USA). We also used double immunostaining with DAB and Vector Blue as colorimetric reagents, with a combination of monoclonal antibodies for α-smooth muscle actin (α-SMA; Dako Corporation), for macrophages (PG-M1, Dako Corporation), and for leukocytes (human leukocyte common antigen antibody (LCA; Dako Corporation) in adjacent tissue sections. After determining the areas for evaluation by simultaneous observation using a multi-headed light microscope, three authors (YN, TS, and HS) independently evaluated immunoreactivity. Scoring of immunoreactivity was performed based on our previous reports with some modifications [10,16]. When PTOV1 protein was immunolocalized to the cytoplasm, the relative immunoreactivity in each specimen was classified into the following three groups: 2 = more than 50% positive cells; 1 = more than 10% and less than 50% positive cells; and 0 = negative or less than 10% positive cells, respectively [16]. When PTOV1 protein immunoreactivity was detected in the nuclei, the relative immunoreactivity in each specimen was evaluated by the percentage of immunoreactivity, ie the labelling index (LI) [10]. When interobserver differences were >5%, the three aforementioned authors re-evaluated these discrepant immunostained slides simultaneously using a multi-headed light microscope, and the mean value was obtained.

Statistical analysis

Values for all results were given as the mean ± standard error of the mean (SEM). Results of quantitative RT-PCR, cell count assay, and the relative immunoreactivity for protein in the nuclei were analysed using one-way analysis of variance followed by unpaired *t*-test for comparisons between two groups. Results of immunohistochemistry of cytoplasmic protein were analysed using the χ^2-test. Statistical differences between immunoreactivity for PTOV1 protein and AR were evaluated using Spearman's rank correlation. A p value <0.05 was considered significant in this study.

Results

Characterization of two VSMC cell lines

By RT-PCR analysis, both HUVS-112D cells and T/G HA-VSMC cells expressed *AR* mRNA (Figure 1A). In addition, AR protein was demonstrated in both of these cell lines by immunoblotting analysis (110 kD) (Figure 1B). Relative levels of AR mRNA and protein expression in these cells were approximately 5–10% of those in LNCaP cells that were examined in parallel (data not shown).

Gene chip microarray assay

Table 3 summarizes 11 genes that were up-regulated by testosterone treatment in both types of VSMC in duplicated microarray analysis. Among these genes, human prostate tumour overexpressed protein 1, ie *PTOV1* was detected in both of these VSMCs. Recently, *PTOV1* has been reported to be induced by androgen and to be involved in cell cycle regulation [14,15,17]. *AGTR2* was also reported to be associated with androgenic effects, but it is unknown whether *AGTR2* is involved in cell growth [18]. Therefore we focused our subsequent studies on *PTOV1* as

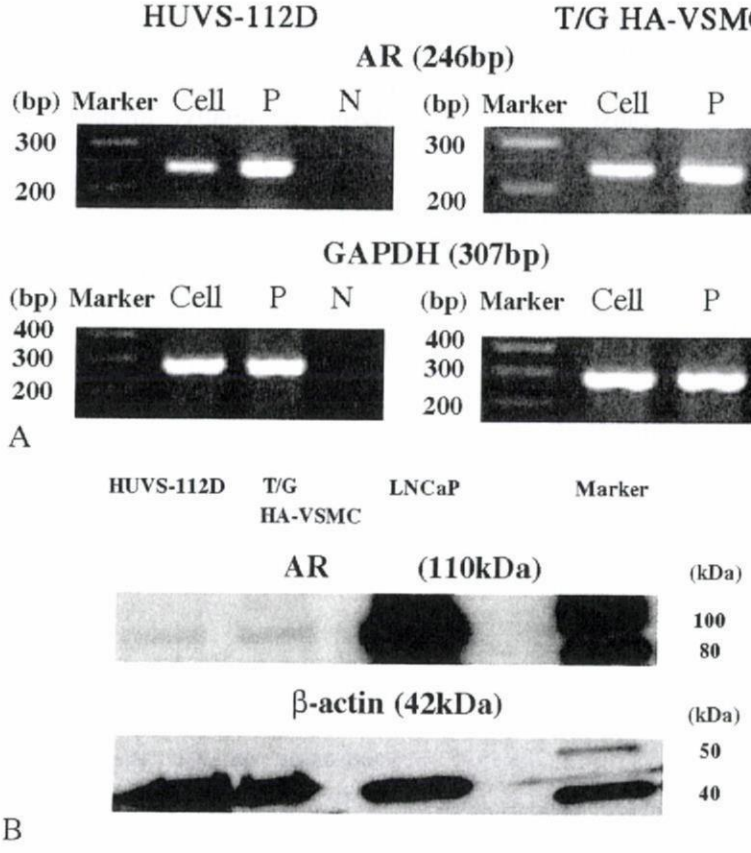

Figure 1. (A) Results of RT/real-time PCR analysis for *AR* and *GAPDH* in two cultured human VSMCs (HUVS-112D, and T/G HA-VSMC), positive controls, and negative controls. Cell = each type of cultured vascular smooth muscle cell; P = positive control (LNCaP prostate cancer cell line); N = negative control (no cDNA), respectively). (B) Immunoblotting analysis of AR and β-actin in HUVS-112D, T/G HA-VSMC, and LNCaP cells. Total protein was extracted, and 60 μg protein from each cell was loaded. Immunoblotting analysis demonstrated both AR (110 kD) and β-actin protein (42 kD) in all cells

Table 3. Ratios of gene expression determined by GeneChip microarray analysis after testosterone treatment of cultured VSMCs for 2 h

Gene symbol	HUVS-112D	T/G HA-VSMC	Function	Association with androgen (reference)
PAK7	3.9	3.6	Neurite development	Unknown
PIK3R4	1.7	3.3	Cell signalling	Unknown
CELSR1	1.9	3.1	Cell adhesion	Unknown
CACNA1G	2.5	2.9	Calcium channel	Unknown
AGTR2	2.2	2.5	Regulator of aldosterone secretion	Koike *et al* [18]
INVS	2.7	3.6	Renal tubular development	Unknown
GPR77	3.5	2.6	Cell signalling	Unknown
CASP10	3.3	2.8	Apoptosis	Unknown
AP4S1	2.1	2.5	Formation of cell structure	Unknown
T1A-2	1.7	2.7	Membrane glycoprotein	Unknown
PTOV1	1.8	2.0	Cell growth/mitogenesis	Benedit *et al* [14]

'Ratios' represent the mean ratios of expression levels of each gene mRNA in duplicate experiments compared with control.

an androgen-responsive gene possibly involved in the proliferation of human VSMCs.

PTOV1 mRNA expression in VSMCs after androgen treatment

Testosterone significantly increased *PTOV1* mRNA levels in AR-positive VSMCs compared with controls in both of these cell lines ($p < 0.05$) (Figure 2). However, testosterone with flutamide, an AR-blocker (100 nM), did not increase its mRNA expression in either of these cells ($p < 0.05$) (Figure 2).

PTOV1 siRNA transfection and cell proliferation assay

Quantitative RT-PCR analysis demonstrated that *PTOV1* mRNA levels were decreased in a dose-dependent manner in the cells transfected with *PTOV1* siRNAs (Figure 3A). After transfection of negative

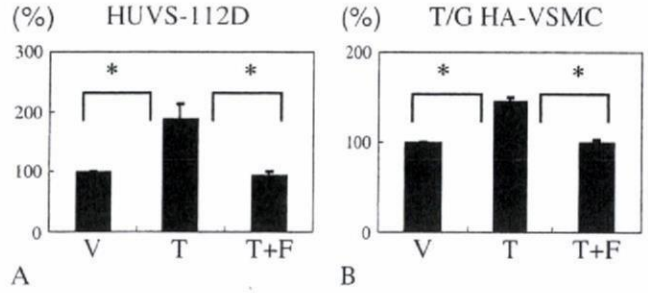

Figure 2. Results of RT/real-time PCR analysis for *PTOV1* in HUVS-112D (A) and T/G HA-VSMC cells (B) among cells treated with vehicle (V) (control), testosterone (T) alone (10 nM), and T (10 nM) with flutamide (F), an AR-blocker (100 nM), respectively after 2 h (*$p < 0.05$)

control siRNA (10 nM), testosterone promoted cell proliferation significantly in both of these cell lines ($p < 0.05$) (Figure 3B). However, testosterone with transfection of *PTOV1* siRNA (10 nM and 100 nM) did not increase cell proliferation in these two cell lines (Figure 3B).

PTOV1 mRNA expression in human aorta

The results of RT/real-time PCR analysis demonstrated the presence of specific single bands for *AR* and *PTOV1* in human aorta (Figure 4A). The relative abundance of *PTOV1* mRNA was significantly greater in male aorta with a mild degree of atherosclerotic changes (group A) than in those of other groups (groups B, C, and D)($p < 0.05$) (Figure 4B). The relative abundance of *AR* mRNA was also significantly greater in male aorta with a mild degree of atherosclerotic change (group A) than in male aorta with a severe

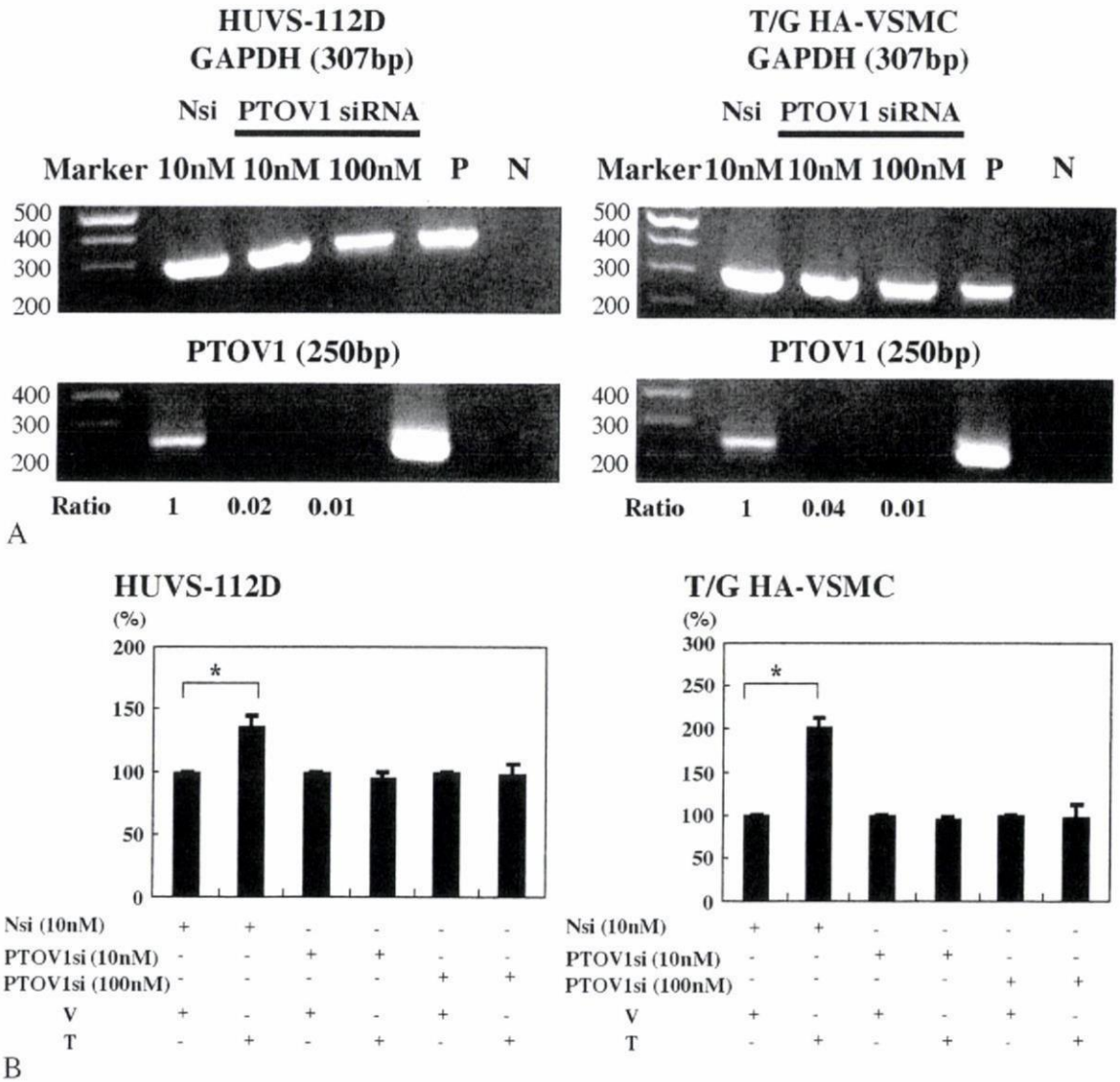

Figure 3. (A) Expression of *PTOV1* and *GAPDH* mRNAs at 24 h after transfection of *PTOV1* siRNA (10 nM or 100 nM) or negative control siRNA (Nsi) (10 nM) in HUVS-112D and T/G HA-VSMC cells detected by real-time PCR, respectively. *GAPDH* mRNA expression was monitored as an internal control. The ratio of *PTOV1*/*GAPDH* was calculated and values were normalized to the ratio obtained from the negative control transfection of Nsi (10 nM). P = positive controls (LNCaP prostate cancer cell lines); N = negative controls (no cDNAs), respectively. (B) The relative levels of cell numbers in HUVS-112D and T/G HA-VSMC cells among cells treated with vehicle (V) (0.1% ethanol) and testosterone (T) alone (10 nM) after transfection of *PTOV1* siRNA (PTOV1si) (10 nM or 100 nM) or negative control siRNA (Nsi) (10 nM) (*$p < 0.05$)

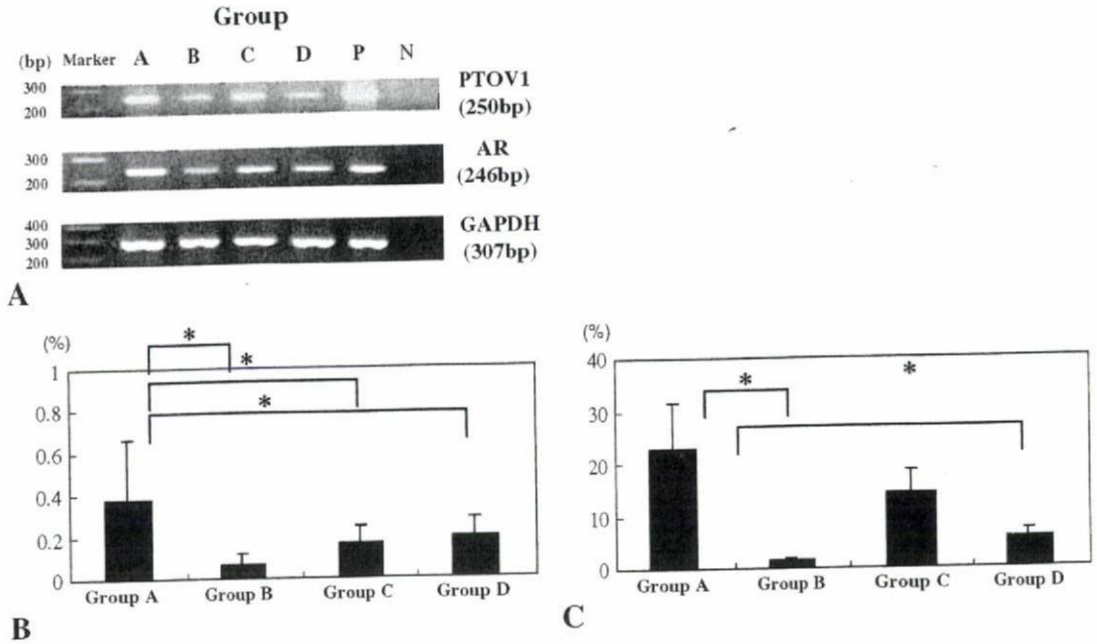

Figure 4. (A) Results of RT/real-time PCR analysis for *PTOV1* in human aortas. Bands for PCR products were detected as specific single bands (246 bp for *AR*, 250 bp for *PTOV1*, and 307bp for *GAPDH*). The amplified products were run on a 2% agarose gel stained with ethidium bromide. Representative photographs for these RT/real-time PCR gene products are illustrated. A = aorta from a 38-year-old man with mild atherosclerotic change; B = aorta from a 72-year-old man with severe atherosclerotic change; C = aorta from a 45-year-old woman with mild atherosclerotic change; D = aorta from a 76-year-old post-menopausal woman with severe atherosclerotic change; P = positive controls; N = negative controls. (B) Results for *PTOV1* mRNA expression levels ($^*p < 0.05$). (C) Results for *AR* mRNA expression levels ($^*p < 0.05$)

degree of atherosclerosis (group B) and in female aorta with a severe degree of atherosclerosis (group D) ($p < 0.05$) (Figure 4C).

Immunohistochemistry for PTOV1 in human aorta

PTOV1 protein was expressed in both the nucleus and the cytoplasm of VSMCs in each group examined (Figures 5 and 6). AR protein was expressed in the nuclei of VSMCs in each group (Figures 5 and 6). However, none of the LCA- or PG-M1-positive cells demonstrated any PTOV1 immunoreactivity (Figure 5). The relative levels of PTOV1 immunoreactivity in the nuclei of neointimal VSMCs were significantly higher in male aorta with a mild degree of atherosclerotic change (group A) than in those of the other groups examined (groups B, C, and D) (Figure 6A). In addition, AR-positive cells in the neointima were also significantly more abundant in male aorta with a mild degree of atherosclerotic changes (group A) than in those of the other groups (groups B, C, and D) ($p < 0.05$) (Figure 6E). There was also a significant positive correlation between AR and PTOV1 immunoreactivity in the nuclei of VSMCs in the neointima ($p < 0.05$) (data not shown). AR-positive cells in the tunica media were significantly more abundant in male aorta with a mild degree of atherosclerotic change (group A) than in male aorta with a severe degree of atherosclerosis (group B) and in female aorta with a mild degree of atherosclerosis (group C) ($p < 0.05$) (Figure 6F). However, there were no significant differences in PTOV1 immunoreactivity in the cytoplasm of cells in the neointima or in the nucleus and/or cytoplasm of cells in the tunica media among these groups (Figure 6B, C, and D).

Discussion

In our present study, results of both microarray and quantitative RT-PCR analyses all indicated that *PTOV1* is one of the genes induced by testosterone via AR-dependent pathways in cultured human VSMCs. In addition, siRNA analysis demonstrated that *PTOV1* is involved in AR-mediated VSMC proliferation. Results of both quantitative RT-PCR and immunohistochemical studies in human aorta obtained at autopsy further demonstrated that PTOV1, as well as AR, detected in the nuclei of neointimal VSMCs was abundant in relatively young male aorta at an early stage of atherosclerosis.

PTOV1 has been known to be involved in stimulation of cell proliferation [14,15,17]. This gene is a mitogenic factor that shuttles between nucleus and cytoplasm in a cell cycle-dependent manner in prostate carcinoma cells [14,15,17]. In addition, *PTOV1* overexpression induced cell proliferation and facilitated entry of prostate carcinoma cells into the S phase [14,15,17]. Therefore, these findings all indicated that *PTOV1* may play a very important role in the proliferation of VSMCs. However, it is also true that other atherogenic effects on human VSMCs, such as promotion of PDGF-induced VSMC proliferation,

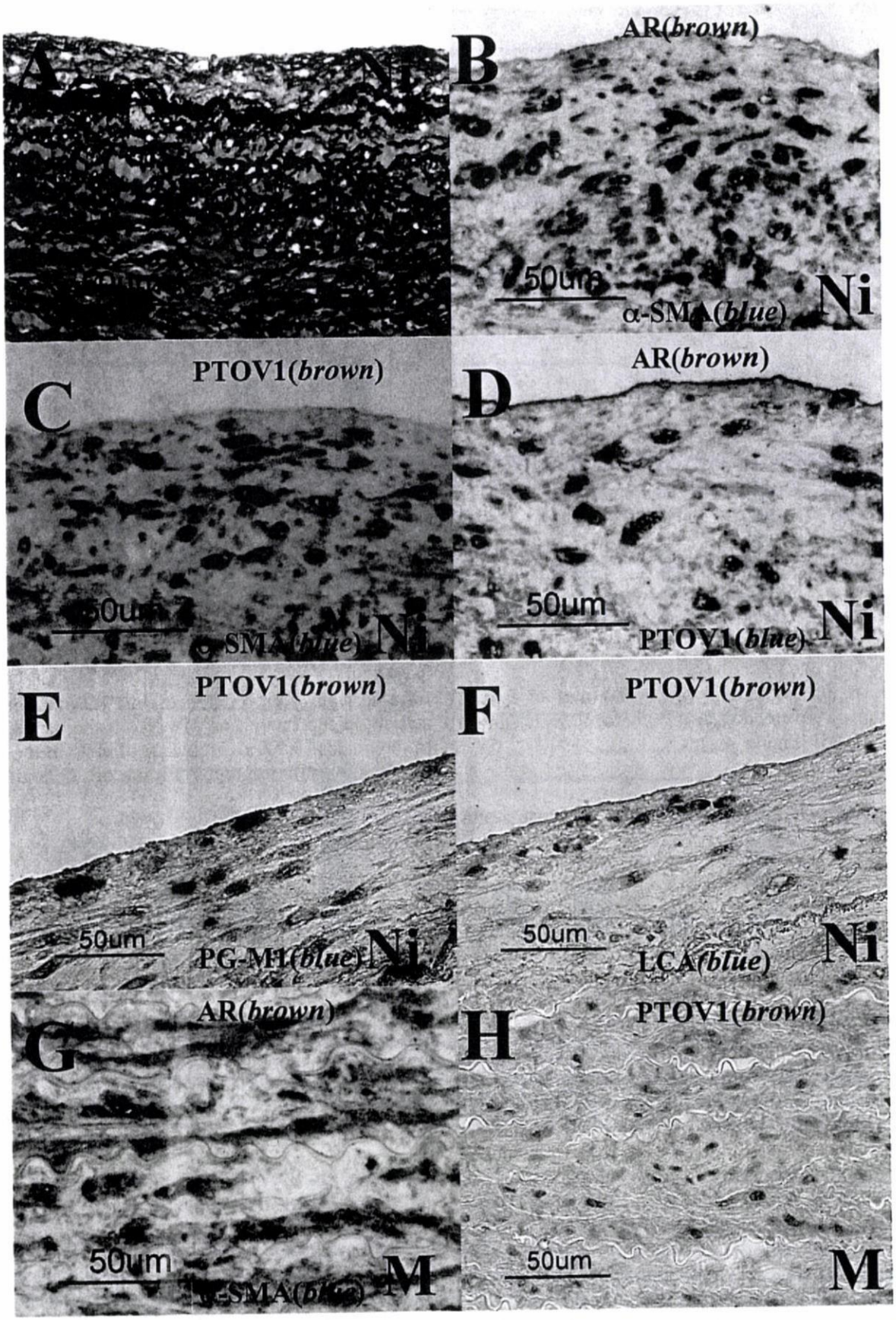

Figure 5. Modified Masson Goldner's stains (A), double-immunohistochemical staining for AR and α-muscle actin (α-SMA) (B), for PTOV1 and α-SMA (C), for AR and PTOV1 (D), for PTOV1 and PG-M1 (E), for PTOV1 and leukocyte common antigen (LCA) (F) in the neointima, double-immunohistochemical staining for AR and α-SMA (G) and immunohistochemical staining for PTOV1 (H) in the media of an abdominal aorta specimen obtained from a 38-year-old man with a mild degree of atherosclerosis (group A). Immunopositive cells appear brown as a result of DAB colorimetric reaction and blue as a result of Vector Blue colorimetric reaction. Double-immunopositive cells are confirmed. Ni = neointima; M = media

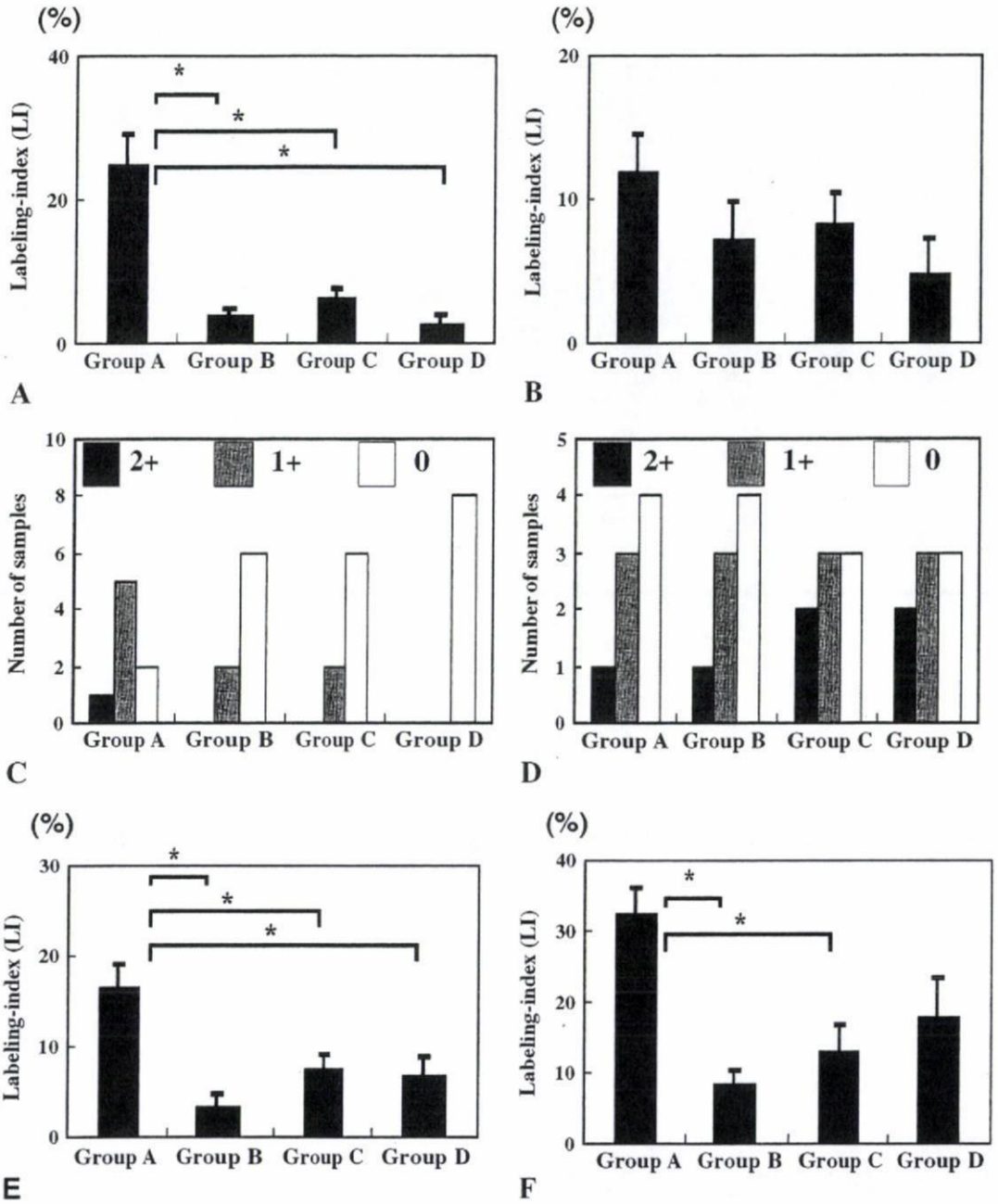

Figure 6. (A, B) The relative immunoreactivity of PTOV1 in the nuclei of VSMCs in the neointima (A) and media (B) was evaluated by the labelling index (LI) in each group (0–100), respectively. Data are the mean ± SEM. $^*p < 0.05$, a significant difference between two groups. (C, D) The relative immunoreactivity of PTOV1 in the cytoplasm of VSMCs in the neointima (C) and media (D) was evaluated by the percentage of positive cells (0, 1+, and 2+) in each group, respectively. (E, F) The relative immunoreactivity of AR present in the nuclei of VSMCs in the neointima (E) and media (F) was evaluated by the LI in each group (0–100), respectively. Data are the mean ± SEM. $^*p < 0.05$, a significant difference between two groups

have been reported as the mechanism for androgen-induced effects [19,20]. Therefore, further investigations are required to clarify how these pathways interact in exerting androgenic effects on VSMC proliferation in the human vascular system. Our present siRNA study demonstrated that *PTOV1* may be involved in testosterone-induced VSMC proliferation. However, further investigation is required to clarify the correlation between PTOV1 expression and testosterone-induced VSMC proliferation by reconstituting *PTOV1* expression after transfection of *PTOV1* siRNA.

Quantitative RT-PCR analysis in our present study also demonstrated that flutamide, an AR-blocker, suppressed androgen-induced *PTOV1* mRNA expression. The chromosomal region where *PTOV1* is located, 19q13.3–13.4, has also been demonstrated to harbour a large number of genes whose expression is modulated by androgens [14]. In addition, the expression of *PTOV1* was reported to be induced by exposure to androgens in LNCaP, an androgen-dependent prostate carcinoma cell line [14,17]. Therefore, these findings all indicate that *PTOV1* should also be considered one of the testosterone-induced genes in AR-positive VSMCs.

We also demonstrated that PTOV1 immunoreactivity in the nuclei of neointimal VSMCs was abundant in relatively young male aorta associated with early stage atherosclerosis. High levels and nuclear localization of PTOV1 have also been associated with cell proliferation in prostate carcinoma cells [14,17]. Neointimal VSMCs are, therefore, considered to play very important roles in the development of atherosclerosis in humans, particularly at an early stage, compared with VSMCs in the tunica media [10,11]. Therefore, higher expression of PTOV1 in these VSMCs is possibly related to the development of atherosclerosis. Levels of PTOV1 and AR were higher in male aorta with mild atherosclerosis than in female aorta with mild atherosclerosis. Men are generally considered to have a higher risk of developing cardiovascular disease than similarly aged women because of prolonged exposure to higher androgen concentrations [19,21]. It has also been shown recently that androgens upregulate atherosclerosis-related genes in macrophages from men, but not from women, which reflects the complexity of gender-related atherogenesis [19,22].

Our present study also demonstrated that the relative abundance of AR and PTOV1 in neointimal VSMCs was significantly higher in younger male aorta with mild atherosclerotic changes than in male aorta with severe atherosclerotic changes. However, these findings appear to contradict the hypothesis that, if PTOV1 is induced by androgens and implicated in androgenic effects on atherosclerosis, its expression should be higher in male aorta with more severe atherosclerosis than in male aorta with mild atherosclerosis owing to a longer exposure to elevated serum testosterone levels. There are two possible reasons for this: firstly, decreased AR and PTOV1 expression in the neointima of male aorta with severe atherosclerosis may be induced by the age-related decrease in serum testosterone levels [23]; and, secondly, when neointimal formation progresses, VSMCs with AR expression become less abundant than those without AR and these cells are therefore not necessarily influenced by androgenic atherogenic effects. Therefore, PTOV1 expression in the neointimal VSMCs in the aortas of men with high serum androgens levels may be associated with the androgen-induced onset of atherosclerosis; this may be important for formation of the neointima in the early stages of atherogenesis in the male aorta. However, recently, low concentrations of testosterone have been associated with an increased risk of cardiovascular disease in men [24]. Androgens are also known to be a coronary vasodilator, and a study of postmenopausal women demonstrated that endogenous androgens correlated inversely with carotid neointimal thickness, which suggests that androgens have potential beneficial effects on the human vascular system [19,25,26]. These different effects of androgens may depend on differences in the androgen-responsive genes induced, but further investigations are required to clarify possible direct androgenic effects on the human cardiovascular system.

In summary, *PTOV1* is considered to be one of the testosterone-induced genes involved in AR-mediated stimulation of VSMC proliferation in the aortic neointima and may play important roles in androgen-related atherogenesis in the male human aorta.

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Memory impairment associated with a dysfunction of the hippocampal cholinergic system induced by prenatal and neonatal exposures to bisphenol-A

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Abstract

One of the most common chemicals that behaves as an endocrine disruptor is the compound 4,4′-isopronylidenediphenol, called bisphenol-A. In the previous study, we reported that exposure to bisphenol-A induced the abnormality of dopamine receptor functions in the mouse limbic area, resulting in a supersensitivity of drugs of abuse-induced pharmacological actions. The present study was undertaken to investigate whether prenatal and neonatal exposures to bisphenol-A could alter other behavioral abnormalities such as anxiogenic behavior, motor learning behavior, or memory. In the present study, adult female mice were chronically treated with bisphenol-A-admixed powder food from mating to weaning. All experiments were performed using male pups. Here we found that prenatal and neonatal exposures to bisphenol-A failed to induce anxiogenic effects and motor-learning impairment using the light-dark test, elevated plus maze test, and rota-rod test. On the other hand, we found that prenatal and neonatal exposures to bisphenol-A induced the memory impairment using the step-through passive avoidance test. Immunohistochemical study showed the dramatic reduction in choline acetyltransferase-like immunoreactivity, which is a marker of acetylcholine (ACh) production, in the hippocampus of mice prenatally and neonatally exposed to bisphenol-A. These results suggest that chronic exposures to bisphenol-A could induce the memory impairment associated with the reduction in ACh production in the hippocampus.

Keywords: Bisphenol-A; Memory; Choline acetyltransferase; Hippocampus; Endocrine disrupting chemicals

Recently, the general public has received alarming reports regarding the reproductive and health hazards of endocrine-disrupting chemicals in the environment. One of the most common endocrine disruptors is the compound 4,4′-isopronylidenediphenol, called bisphenol-A, which is used in the manufacture of many types of products.

Our recent studies suggest that exposure to bisphenol-A during prenatal and postnatal development has long-lasting effects on central dopaminergic systems linked to rewarding effects, as well as drug addiction induced by drugs of abuse [13–16,21,22]. The fetus uses natural hormonal messages that originate in its own hormone system and that of its mother for developmental guidance. Although bisphenol-A, has weak estrogenic activity, prenatal and neonatal exposures to 17β-estradiol failed to induce the supersensitivity of morphine [13]. Furthermore, although it is well known that bisphenol-A disrupts thyroid hormone, prenatal and neonatal exposures to propylthiouracil, an inhibitor of thyroid hormone synthesis, reduced the activation of dopaminergic neurons (unpublished observation). These findings indicate that the disruption of dopaminergic neuron development induced by prenatal and neonatal exposures to bisphenol-A can be mediated by nonhormonergic actions of bisphenol-A. Furthermore, our preliminary biochemical studies showed that bisphenol-A has an affinity for nonspecific binding sites. Thus since its action site remained unclear, it is most likely that prenatal and neonatal exposures to bisphenol-A induce other behavioral abnormalities associated with the alternation not only of the dopaminergic system, but also of other neurotransmissions. The present study was then undertaken to investigate whether prenatal and neonatal exposures to bisphenol-A could alter other behavioral abnor-

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