vitro. However, several important questions still remain to be answered. In particular, both GPR40 and GPR120, whose natural ligands have similar pharmacological properties, are expressed in the intestinal tract and in STC-1 cells, but only the stimulation of GPR120, not GPR40, leads to the secretion of gut peptides. To convincingly demonstrate the contribution of these receptors in fat-stimulated CCK secretion, development of GPR40- or GPR120-selective ligands, and/or genetically engineered animals (transgenic and/or knockout mice) would be required for further studies.

Acknowledgements This work was supported in part by research grants from the Scientific Fund of the Ministry of Education, Science, and Culture of Japan (to G.T., T.A., and T.-a.K.); the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) (to G.T., A.H., T.A. and T.-a.K.); and the 21st Century COE Program "Knowledge Information Infrastructure for Genome Science"(to G.T., T.A. and T.T).

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Production and characterization of a monoclonal antibody against GPR40 (FFAR1; free fatty acid receptor 1)

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Received 14 October 2007
Available online 31 October 2007

Abstract

GPR40 is G protein-coupled receptor whose endogenous ligands have recently been identified as free fatty acids (FFAs), and it has been implicated to play an important role in FFA-mediated enhancement of glucose-stimulated insulin release. We have developed a monoclonal antibody against the extracellular domain of GPR40. Specificity of the antibody was demonstrated by immunoprecipitation and cell surface staining using GPR40-transfected cells. GPR40 immunoreactivity was highly abundant in mouse pancreatic β-cells and splenocytes, THP-1 cells, and human peripheral blood mononuclear cells. The anti-GPR40 monoclonal antibody should prove valuable for further studying the function of this nutrient sensing receptor.
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Keywords: GPR40; Free fatty acid receptor 1; Flow cytometry; Monoclonal antibody; Monocytes; THP-1 cell

Free fatty acids (FFAs) are not only essential nutritional components, but they also function as signaling molecules. Recently, a G protein-coupled receptor (GPCR) de-orphanizing strategy successfully identified multiple receptors for FFAs, which function on the cell surface and play significant roles in the regulation of metabolism [1–4]. GPR40 has been reported to be a receptor for medium to long-chain fatty acids, which are abundantly expressed in pancreatic β-cells, and it also plays a significant role in the chain of events linking obesity and type 2 diabetes [5]. GPR120, a GPCR that prefers long-chain FFAs as natural ligands and that is abundantly expressed in lung, intestinal tract, and adipocytes, was also recently identified [4,6].

Since neither a selective ligand nor a radiolabeled ligand is currently available for either of these long-chain free fatty acid receptors (FFARs), the corresponding receptor transcripts have been detected to characterize the tissue distribution of each FFAR expression. GPR40 mRNA is expressed primarily in the pancreas, brain, and monocytes [1]. It is, however, suggested that the level of mRNA expression in a given tissue may not directly correlate with the level of the receptor protein it encodes [7]. Thus, it is important to determine the expression profile of each receptor subtype protein.

In the present study, we have generated and characterized monoclonal antibodies directed to the extracellular domain of the fatty acid receptor, GPR40. We have also developed an immunohistochemical and flow cytometry analysis protocol that allows efficient detection of GPR40 proteins in native tissues and cells. The generation of this novel antibody enabled us to determine the cellular and

Abbreviations: GPCR, G protein-coupled receptor; FFA, free fatty acid; FFAR1, free fatty acid receptor 1.

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0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.10.142

subcellular distribution of GPR40 proteins in a variety of human and mouse tissues.

Materials and methods

Generation of monoclonal antibodies. Peptides were synthesized corresponding to amino acids 62–76 (peptide: GPR40-EC2; KAVEALASGAWPLPL(C)) of the human and mouse GPR40 (Fig. 1A). Peptide GPR40-EC2 was conjugated through an added carboxyl-terminal cysteine to the activated carrier protein KLH (Calbiochem, San Diego, CA, USA), according to the MBS protocol (Pierce, Rockford, IL, USA). The conjugated peptide (200 μg/mouse) was emulsified in complete Freund's adjuvant (Sigma, St. Louis, MO, USA) and injected subcutaneously into 5–12 week-old GANP/B6 mice [8]. Mice were boosted four times with 50 μg/mouse of the peptide emulsified in incomplete Freund's adjuvant (Sigma). Animals were sacrificed three days after the last injection and splenocyte suspensions were purified by Cell strainer (Falcon BD, San Jose, CA, USA), then fused at a 1:5 ratio with the mouse P3U1 (P3) hybridoma fusion partner using standard techniques [9]. Hybridomas were selected in complete RPMI1640-10% FCS with 1× HAT supplement, prior to limiting dilution culture in 96-well plates (15 plates per fusion) for seven days. After HAT selection, hybridomas were passaged into 1× HT medium for five days, during which time supernatants were collected in 96-well format and screened by ELISA.

cDNA construct, cell culture, and transfection. Cell culture for HEK293 cells was performed as described previously [10]. Human monocytic THP-1 cells were purchased from Riken Cell Bank (Tsukuba, Japan) and cultured in RPMI 1640 medium (Gibco, Grand island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) in a humidified 5% CO_2 incubator at 37 °C. Human GPR40 cDNA was obtained by PCR using genomic DNA as a template and ligated into the multicloning site of mammalian expression vector pcDNA5/FRT/TO (Invitrogen Japan, Tokyo, Japan) with the N-terminal FLAG tag.

Immunoprecipitation and Western blot analysis. Cells were solubilized with 0.5% digitonin/PBS on ice for 1 h. Lysates were centrifuged in a microfuge for 10 min at 10000×g two times to sediment the insoluble material. The soluble fraction was immunoprecipitated by anti-GPR40 antibody or anti-FLAG antibody as described previously [11]. Nonspecific immunoprecipitation was assessed using the antibody plus excess antigenic peptide. Immunoprecipitates were detected by Western blot analysis with anti-FLAG antibody using Western Lightning Chemiluminescence Reagent Plus kit (Perkin–Elmer, Boston, MA, USA).

Flow cytometry analysis. For the flow cytometry analysis using antibodies, attached cells were trypsinized and washed twice with PBS. Cells were then incubated for 45 min at 4 °C with anti-GPR40 antibody (1 μg/ml) in PBS including 1% BSA, washed with the same buffer, and incubated with FITC-conjugated anti-mouse IgG (ICN Biomedicals, Solon, OH, USA) for 45 min at 4 °C. Analysis of the cells was performed using a FACSCalibur (BD Biosciences, San Jose, CA, USA) as described previously [11].

Immunocytometry and immunohistochemistry. For immunocytometry, HEK293 cell lines were grown on glass-base dishes (Asahi Techno Glass, Tokyo, Japan). Transfected cells were fixed in 4% paraformaldehyde/PBS for 5 min, and permeabilized in 0.1% Triton X-100/Hepes/DMEM for 3 min. Immunostaining using anti-GPR40 antibody was performed as described previously [11].

For immunohistochemistry, Male C57/6 mice, kept on a standard laboratory diet, were anesthetized with pentobarbital intraperitoneally and fixed by vascular perfusion. The fixative contained 2% paraformaldehyde, 66.6% saturated picric acid solution, and 50 mM phosphate buffer. The pancreases and spleen were removed. After fixing in the same fixative for more than 4 h, samples were immersed in 20% sucrose dissolved in 0.1 M phosphate buffer overnight and then frozen in liquid nitrogen. The tissues were stored at −80 °C until use. Ten micrometer thick sections were cut in cryostat and mounted on glass slides. After Avidin/Biotin Blocking (Vector laboratories, Burlinghame, CA, USA), sections were incubated for 30 min at RT and overnight at 4 °C in a humidified chamber with M.O.M. mouse Ig Blocking Reagent (Vector laboratories). After M.O.M. Diluent (Vector laboratories) for 5 min, guinea pig anti-mouse insulin antibodies (1 μg/ml, Abcam, Cambridge, UK) were incubated for 30 min and rhodamine conjugated anti-guinea pig IgG (2.5 μg/ml, CHEMICON, Temecula, CA, USA) for 1 h at RT. Next anti-GPR40 antibodies (1 μg/ml) were incubated for 30 min and M.O.M. biotinylated anti-mouse IgG reagent (1:1000, Vector laboratories) for 10 min and added Fluorescein Avidin DCS (1:62.5, Vector laboratories) for 5 min each at RT. Sections were mounted in Prolong Gold antifade reagent (Invitrogen) and observed using a confocal laser scan microscopy system FLUOVIEW FV1000 (Olympus, Tokyo, Japan).

Preparation of splenocytes, PBMC, and CD14+ PBMC. For preparation of spleen cells, phosphate-buffered balanced salt solution (PBBS) supplemented with 0.1% BSA was used as described [12]. To remove erythrocytes, spleen cells were treated with the Tris-buffered ammonium chloride solution and then rinsed three times with PBBS.

Whole blood was drawn by clean venipuncture from healthy volunteers. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood using Lymphoprep tubes (Axis-Shield PoC, Norton, MA, USA). After washing with PBS, CD14+ cells were separated from the PBMC by positive selection using CD14+ micro magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's protocol.

Results and discussion

Characterization of the anti-GPR40 antibody

A monoclonal antibody (named EC2_G16) was obtained, and its specificity was monitored using immunoprecipitation, flow cytometry, and immunohistochemistry. FLAG-tagged GPR40-transfected cells were solubilized, immunoprecipitated by anti-GPR40 antibody or anti-FLAG antibody, and electrophoretically separated and blotted onto PVDF membranes. Immunodetection with the anti-FLAG antibody revealed an approximately 30 kDa band in immunoprecipitations carried out with the anti-FLAG or anti-GPR40 antibody in doxycycline-induced FLAG-tagged GPR40 cell lysates (Fig. 1B). The 30 kDa band was not detected in the uninduced cell lysates or after addition of excess immunogen peptide GPR40-EC2 (10 μg/ml). The antibody was further characterized using flow cytometry analysis of transiently transfected cells. After transfection with GPR40 cDNA, the HEK-293 cells were stained by either the anti-FLAG antibody or the anti-GPR40 antibody, and then flow cytometry analysis was performed (Fig. 1C). After transfection, approximately 9–12% of the HEK-293 cells were positively stained by either the anti-FLAG antibody or the anti-GPR40 antibody. In contrast, no cells were positively stained by either the anti-FLAG antibody or the anti-GPR40 antibody when transfected with the control vector (data not shown). The antibody was further characterized by performing immunofluorescent staining of transfected cells. When HEK-293 cells transiently expressing GPR40 were stained with anti-GPR40 antibody, a prominent immunofluorescent signal was detected that was localized at the plasma membrane (Fig. 1D). These results indicate that the anti-GPR40 antibody EC2_G16 specifically detected the GPR40 protein.

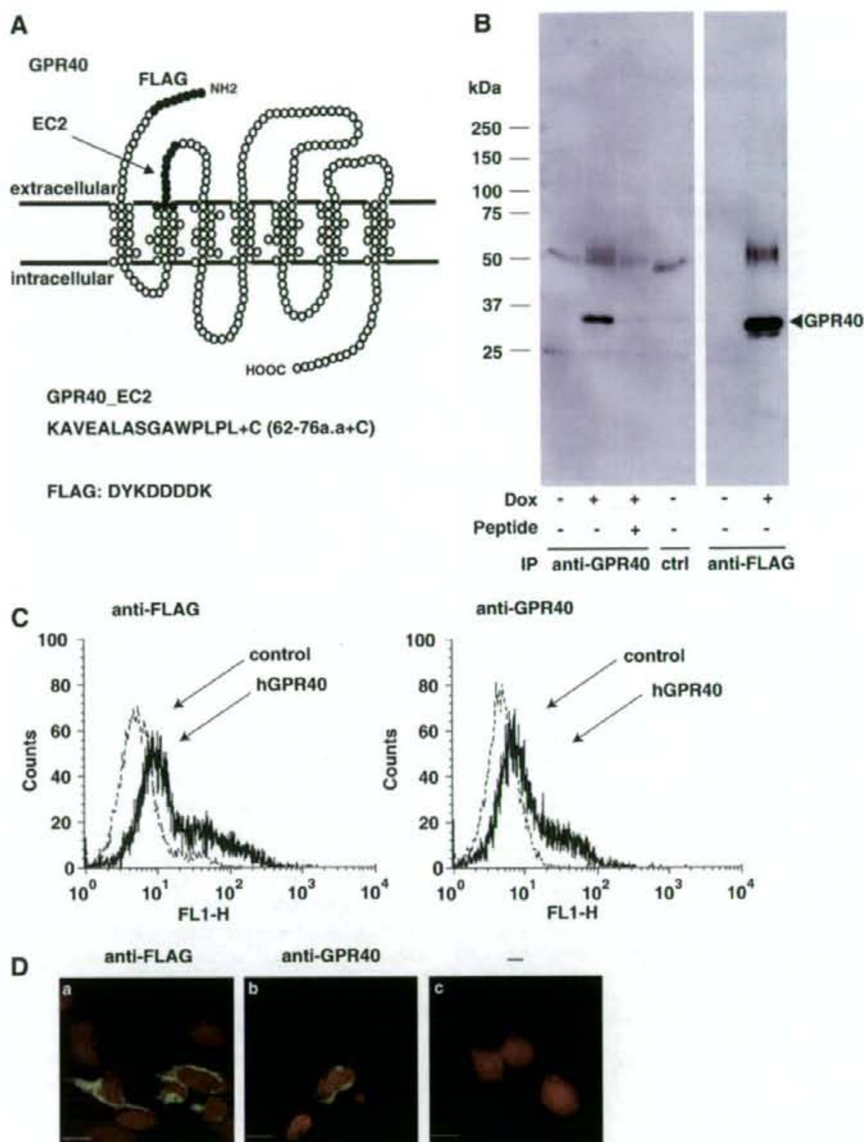

Fig. 1. Characterization of the anti-GPR40 monoclonal antibody in GPR40-transfected HEK cells. (A) Schematic diagram of GPR40. Following the generally accepted model of G protein-coupled receptors, the seven highly homologous hydrophobic regions are shown as membrane-spanning domains. The amino acid sequences of GPR40-EC2 (mouse and human) and the FLAG epitope are shown. (B) Immunoprecipitation and Western blot analysis of the anti-GPR40 antibody. FLAG-tagged, GPR40 inducibly transfected HEK293 cells were solubilized, immunoprecipitated by the anti-GPR40 antibody, control antibody or anti-FLAG antibody, and electrophoretically separated and blotted onto a PVDF membrane. Blots were developed using the anti-FLAG antibody and enhanced chemiluminescence. Two additional experiments gave similar results. Ordinate, migration of protein molecular weight markers ($Mr \times 10^{-3}$). (C) Flow cytometry analysis of hGPR40-expressing cells. HEK293 cells transfected with human FLAG-tagged GPR40 (hGPR40) or a control vector (control) were analyzed using a FACSCalibur flow cytometer. The cells were stained with an anti-FLAG antibody (left) or an anti-GPR40 antibody (right) and FITC-labeled secondary antibody. (D) Characterization of the anti-GPR40 antibody by immunofluorescent staining of transfected cells. HEK293 cells transfected with human FLAG-tagged GPR40 were subsequently fixed and stained immunofluorescently with the anti-FLAG antibody (a), the anti-GPR40 antibody (b) or without the 1st antibody (c). Green; FITC, Red; TO-PRO-3. Scale bar, 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

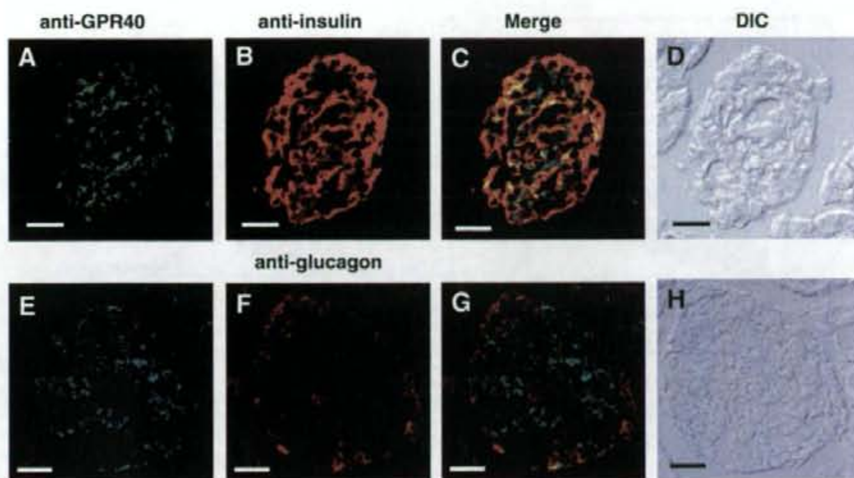

Fig. 2. Cross-staining of GPR40, insulin, and glucagon immunoreactivities. Confocal microscopy images of mouse pancreas, showing staining for GPR40 (A, E), insulin (B), glucagon (F), Merge (C, G), and DIC (D, H). Representative results from one of the three independent experiments are shown. Scale bar, 20 μm.

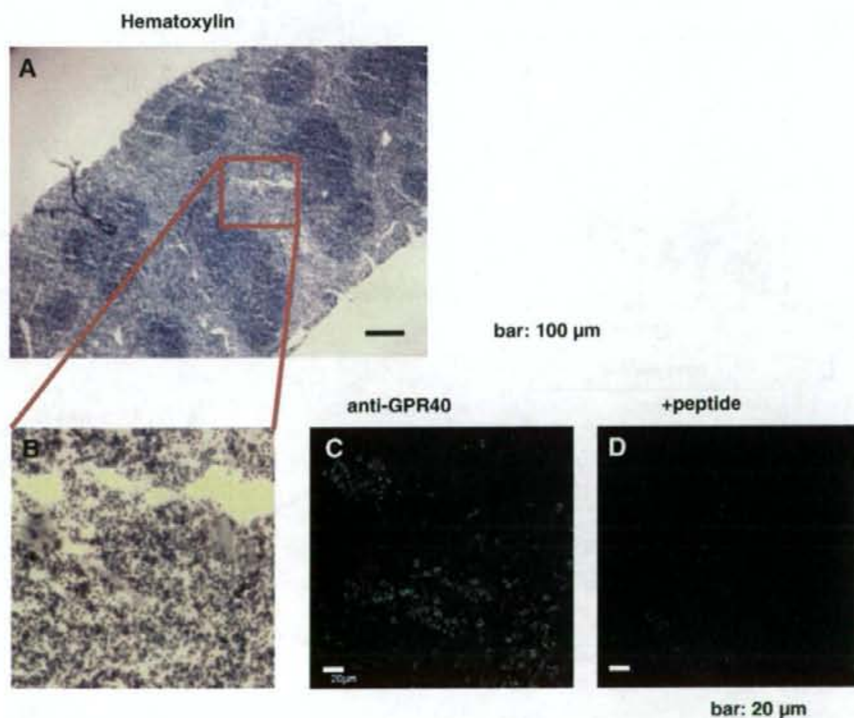

Fig. 3. Hematoxylin (A, B) and GPR40 immunohistochemical staining (C, D) in mouse spleen. For adsorption controls, the primary antibody was incubated with 10 μg/ml of the peptide used for immunizations. Representative results from one of the three independent experiments are shown. Scale bar, 20 μm.

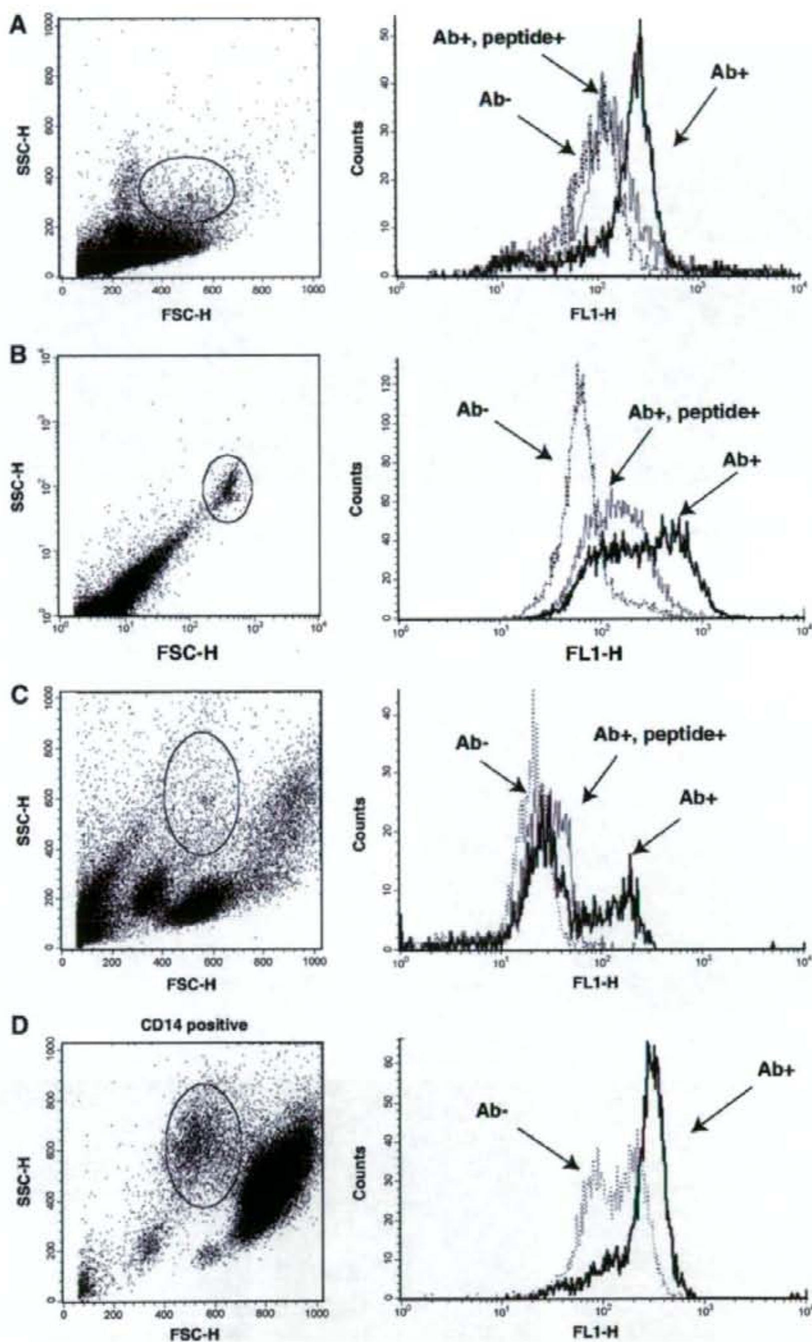

Fig. 4. Flow cytometry analysis of THP-1 cells and freshly isolated native cells. Freshly isolated mouse spleen cells (A), THP-1 cells (B), human PBMC (C), and human CD14-positive PBMC (D) were stained with the anti-GPR40 antibody (Ab+), the antibody with the immunogen peptide (+Peptide) or without the first antibody (Ab−), followed by the FITC-labeled secondary antibody, and analyzed on a FACSCalibur flow cytometer. The gated cells (circled in the FSC–SSC plot of the left panel) were selected and are shown as a histogram in the right panel.

Immunological analysis of native tissues and cells

Using the anti-GPR40 antibody EC2_G16, we further examined the native cells and tissues previously shown to express GPR40 mRNA. First, with the anti-GPR40 antibody, we performed the immunohistochemical staining of pancreatic islets, where GPR40 mRNA expression and immunoreactivity were previously reported [3,13]. Confocal immunofluorescence microscopy with either an anti-insulin antibody, an anti-glucagon antibody (labeled with rhodamine) or EC2_G16 antibody (labeled with FITC) showed that the GPR40 is clearly coexpressed with insulin in intact islet cells; however, on the other hand, we could barely detect cells showing both GPR40 and glucagon immunoreactivity (Fig. 2).

Next, we characterized mouse spleen, where GPR40 mRNA expression was detected by RT-PCR [6]. Staining with the anti-GPR40 antibody indicated that GPR40 immunoreactivity was predominantly confined to the subpopulation of cells in the red pulp of the mouse spleen (Fig. 3). This immunostaining was completely abolished by pre-adsorption of the EC2_G16 antibody with 10 μg/ml of its immunizing peptide GPR40-EC2.

We further characterized the immunostained cells by flow cytometry, using anti-GPR40 antibody. As shown in Fig. 4A, flow cytometry analysis shows that a small population of cells isolated from mouse spleen expresses GPR40 protein. We also analyzed an acute monocytic leukemia cell line, THP-1, which was previously reported to express GPR40 mRNA [1], and found that approximately half of the cells examined were positively stained by the EC2_G16 antibody (Fig. 4B). Furthermore, we found that a small population of cells prepared from human PBMC were positively stained by the EC2_G16 antibody (Fig. 4C). When CD14-positive cells were collected from the human PBMC, GPR40-positive human PBMC were found to be enriched (Fig. 4D). The CD14- and GPR40-positive cells were positively stained by other monocyte markers CD11b, CD33, and CD64 (data not shown), indicating that the PBMC expressing GPR40 proteins are monocytic cells.

It is well established that GPR40 acts as a nutrient-sensing receptor of pancreatic islets; however, its precise cellular and subcellular localization in other tissue(s) have not been fully characterized. We have generated a monoclonal antibody specific for GPR40. We have also shown that the extracellular domain of GPR40 can serve as an epitope to generate an antibody that successfully detects GPR40 protein. The specificity of the antibody was demonstrated by (1) immunoprecipitation of membranes from transfected cells, in which the anti-GPR40 antibody was detected as a band migrating at 30 kDa, (2) the anti-GPR40 antibody revealed prominent cell surface staining of GPR40-transfected cells, and (3) the anti-GPR40 antibody specifically identified cell surface expression of GPR40 in transfected cells. Immunostaining of the anti-GPR40 antibody was completely abolished by pre-adsorption with excess immunogen peptide.

The anti-GPR40 antibody is applicable not only to immunohistochemical detection but also to flow cytometry in native tissues or cells. Our confocal immunofluorescence microscopy analysis showed that the distribution of GPR40 within the pancreatic islet overlapped to great extent with that of insulin. This finding is in good agreement with previous in situ analyses [1,3] and with immunohistochemical studies carried out with a polyclonal-antibody [14]. Using the monoclonal antibody, we could detect GPR40 proteins in the subpopulation of cells in the red pulp of spleen and in the human acute monocytic leukemia cell line THP-1, where GPR40 mRNA was detected [1]. Further, we found for the first time that GPR40 is expressed in a small population of monocytic cells. Further studies will be required to clarify the physiological role of GPR40 in these cells.

In conclusion, we have generated and extensively characterized an anti-GPR40 monoclonal antibody, EC2_G16, whose epitope is the extracellular domain of GPR40. This anti-GPR40 monoclonal antibody should prove useful for further studies of the function of this nutrient sensing receptor.

Acknowledgments

This work was supported in part by a research grant from the Scientific Fund of the Ministry of Education, Culture, Sports, Science and Technology of Japan (to A.H., T.-a.K., G.T.); the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) (to G.T., A.H., T.-a.K.); the 21st Century COE Program "Knowledge Information Infrastructure for Genomic Science" (to G.T.); the Ajinomoto Amino Acid Research Program (to G.T.); the ITOH TOKUZOH Himashi Research funds (to A.H.) and the Takeda Science Foundation (to A.H.).

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資料5

トキシコゲノミクス研究の臨床への展開

藤村　昭夫

自治医科大学 医学部 臨床薬理学

これまで我々は、日本人における医薬品の安全性を予測する新しいシステムを構築するために、トキシコゲノミクス手法を用いた臨床研究を行って来た。

第1ステップとして、手術によって切除された腎がん組織の非病変部よりプライマリー腎細胞を作成し、これを用いてトキシコゲノミクス研究を行う基盤を整備した。その結果、手術検体からプライマリー腎細胞を培養することが出来た。次いで、これらのプライマリー腎細胞を用いて DNA チップによる網羅的遺伝子発現解析を行い遺伝子発現における個人差を検討したところ、解析された 44,000 トランスクリプトの中で、個人差がある（有意水準 p=0.01 以下）と判断されたトランスクリプトは 63 であった。一方、外国から輸入されたヒト腎細胞の遺伝子発現を解析したところ、343 トランスクリプトの発現量が日本人と大きく異なることが明らかになった。次いで、臨床の場で重篤な腎障害の出現することが報告されている薬物をヒトプライマリー腎細胞に曝露させた後に網羅的遺伝子発現データを収集し、これらを解析して腎障害を予測するためのバイオマーカーの検索や腎障害の発現機序の解明を試みた。その結果、急性白血病治療薬として用いられている三酸化ヒ素の腎障害は酸化ストレスの亢進に基づくものであり、この有害作用の軽減薬として抗酸化作用を有しているα一リポ酸が有効であることを示す成績が得られた。さらにラットに三酸化ヒ素を反復投与し、α一リポ酸の併用効果を検討したところ、α一リポ酸は三酸化ヒ素投与によるラットの死亡を防止することが明らかになった。

第2ステップとして、患者を対象としたトキシコゲノミクス研究を実施することを想

定して、末梢血リンパ球を用いたトキシコゲノミクス研究の基盤を整備した。最初に、末梢血からサンプルを調整する際にリンパ球の遺伝子発現に影響を与える可能性のある因子を検討したところ、抗凝固薬としてヘパリンを用いると数個の遺伝子の発現が抑制されることが明らかになり、遺伝子発現解析を目的とした臨床研究ではヘパリンの使用は避けた方が良いと判断された。次いで、埋状第三大臼歯（親不知）を抜歯する予定の患者を対象にして、抜歯前および抜歯・抗菌薬反復投与後に採血し、末梢血リンパ球を分離した。得られた検体から RNA を抽出し、その質を評価したところ、検体を採取してから処理するまでの時間経過と供に、RNA の質が低下することを確認した。さらに、抗菌薬を投与することによって、リンパ球の遺伝子発現が多数変化したことより、トキシコゲノミクス研究を臨床で実施する際に、末梢血リンパ球を用いることは可能だと思われた。次いで、重篤な有害反応として急性腎不全が報告されている薬物 80 種類、および急性腎不全が報告されていない薬物 80 種類を選び、日本人由来不死化 B 細胞に曝露して網羅的遺伝子発現解析を行った。その結果、両群間で異なる発現変動を示した 32 遺伝子を見出し、これを用いて 83%の精度で腎障害性を予測することの出来る腎障害性判定法を確立した。

第 3 ステップとして、現在、自治医科大学附属病院の 4 診療科と共同でトキシコゲノミクス研究を進めている。重篤な有害反応として腎障害、肝障害あるいは間質性肺炎が報告されている 8 種類の薬物を対象にして、これらの薬物を投与する前、および反復投与した後に患者から末梢血を採取し、これを用いて遺伝子発現データを蓄積している。さらに、これらの薬物を動物に投与し、末梢血細胞を用いて遺伝子発現解析を行い、ヒトと動物間のブリッジングを試みる予定である。

P-143 トキシコゲノミクス手法を用いた薬物肝毒性予測の試み

Attempt to prediction of drug-induced liver toxicity using toxicogenomics

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臨床の場で薬物を使用中に肝障害が出現し、治療に支障を来す事がある。これは、前臨床の段階で毒性が詳細に調べられているが、それでもヒトに投与した時に毒性が無いと完全には予測出来ない事が一因である。発ガン性物質や抗がん剤等の毒性の高い化合物に関しては、トキシコゲノミクス手法を利用して、高精度で毒性予測が可能であることが報告されている。また我々は、同じトキシコゲノミクス手法により、腎障害性薬物に関して80%以上の精度で予想することに成功している。そこで今回、腎障害性薬物予測と同じ手法で肝障害性薬物の予測が可能か否かを試みた。薬物曝露する細胞は、将来的に臨床試験にも応用する事を考え、ヒトB細胞由来細胞株であるHEV0034 (RIKEN)とした。肝障害の有無は薬物に関する添付文書情報を参考にして分類し、臨床で認められている最高血中薬物濃度で24時間曝露した細胞からRNAを抽出した。得られたRNAは OVATION Biotin RNA Amplification and Labeling System Ver.1.0 (NuGEN, MediBic)を用いてcDNAの合成とラベリングを行った。ラベル後のcDNAはGeneChip HG-U133 A2.0(Affymetrix)により発現データを取得し、得られた発現データの解析にはGeneSpring GX(トミーデジタルバイオロジー)を用いた。肝障害あり、なし、それぞれをtraining set群、test set群の二つに分け、training setによりそれぞれの薬物で発現の変動のあった遺伝子の分類を試みた。その結果、P値＝0.01で2倍以上の変動のあった53遺伝子が分離された。この遺伝子群を用いてサポートベクターマシーン法を用い、training setで予測精度が最大になる様にマージンを求めた。これをtest setに適用したところ、51サンプル中37のサンプル、すなわち約73%の精度で肝障害の有無を予測する事が出来た。今後はさらに多くの薬物を用いて遺伝子発現データの取得を行い、判別法を含めて、より詳細に検討する予定である。

P-144 Percellome手法を用いたフタル酸エステルDEHPとその活性代謝産物MEHPの腎に及ぼす遺伝子発現変動の比較

Comparison of gene expression profiles on kidney induced by phthalic ester, DEHP, and its active metabolite, MEHP, using the "Percellome" system

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従来、フタル酸ジ-2-エチルヘキシル(DEHP)誘発毒性は、その活性代謝産物モノ-2-エチルヘキシル(MEHP)により誘発されるものと考えられてきた。しかし、マウスを用いた28日間強制経口投与実験において、MEHPはDEHPと比較して、肝、精巣よりも腎標的性が高いことを見いだした(本学術年会、今井らの発表参照)。そこで、このMEHP誘発腎障害機序をより詳細に把握することを目的として、両物質単回投与時の腎に及ぼす遺伝子発現変動を比較・解析した。

12週齢の雄性C57BL/6CrSlcマウスを使用し、DEHPおよびMEHPを単回強制経口投与した。実験は両物質ともに、4時点(投与2、4、8、24時間後)、4用量(DEHP: 0、200、700、2,000 mg/kg; MEHP: 0、70、200、700 mg/kg)、各物質あたり計16群、各群3匹で検討した(溶媒:コーンオイル)。採取した腎のmRNAについてGeneChip MOE430v2(affymetrix社)を用いて、約45,000プローブセットの遺伝子発現の絶対量をPercllome手法により得て、遺伝子発現変動解析をおこなった。その結果、両物質に共通して、核内受容体PPARα受容体及びPPARγ受容体のシグナルカスケードに関係する遺伝子群の発現増加が認められた。また、DEHPでのみ変動が認められたものとして、ミトコンドリア電子伝達系の複合体Iを構成する遺伝子群の軽微な発現増加が認められた。他方、MEHPでのみ変動が認められたものとして、スフィンゴシン-1-リン酸シグナルカスケードに関係する遺伝子群の発現増加が認められ、このシグナルがMEHPの腎選択性に関与する可能性が示唆された。

ポスター　Poster

4P−1188

薬物の腎障害性予測におけるin vitro遺伝子発現解析の施設間バリデーション

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Validation between facilities of in vitro gene expression profile to predict for renal failure of medication

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医薬品開発において、薬の副作用、特に有害反応の予測が問題となり、開発の遅延や中止になる薬が少なくない。そこで薬物有害反応をより早期かつ正確に予測することが必要となる。動物への投与実験では、時にヒトと違う反応も出ることがあり、ヒトへは完全に外挿出来ていないのが現状である。我々は、昨年度の本学会において、in vitroで薬物を曝露し、遺伝子発現データを解析することにより、薬物による腎障害の有無を高精度に予測出来ることを報告した。しかしこの方法が一施設でしか適用出来なければ、汎用性、有用性に乏しく、他の施設で得られた遺伝子発現データを用いても、同じ結果が得られなければならない。そこで、本予測法におけるプロトコールの整理と問題点の確認、そして有用性を確認するために、他施設に試験を依頼し、発現データの取得を行った。今回使用する細胞、RNA抽出キット、cDNAラベリングキット、そしてチップは共通とし、本施設より送付した。細胞は、本施設にて同一日に凍結した細胞を用いた。また曝露薬物は腎障害予測法を確立した際に使用した薬物より、腎障害あり、なしの薬物をそれぞれ5つ、合計10薬物をランダムに選び、試薬類と共に送付した。細胞の継代法と薬物の曝露濃度のみを指定して、実験開始の時期、継代の回数、曝露のタイミング、RNA抽出等は各施設の自由とした。その結果得られた発現データを送付してもらい、当施設にて標準のnormalization後解析を行った。GeneChipにより得られた情報より、cDNAの合成とラベリング、そしてハイブリダイゼーションの効率をチェックしたところ、今回比較を行った5施設の中で、一施設で外れ値を示したが、極端な外れ値ではなく、全ての施設において基準内の品質を保持していることが解った。また、normalizationに用いるhouse keeping geneの発現を比較したところ、有意な差はなく、各施設にて得られた発現データは比較しても問題の無いことが明らかとなった。

P1L-A14-1 Microarray profiling identified a molecular signature associated with ritodrine-induced liver toxicity

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Ritodrine, a β2-sympathomimetic drug used for the treatment of premature labor, has been reported to cause the elevation of liver enzymes such as AST and ALT in pregnant women. β2-stimulation in the liver is also known to cause glycolysis and lipolysis. These imply the liver function could be altered by Ritodrine, but the mechanism under these adverse effects remains largely unknown. In order to identify the factors which induce this liver damage, we constructed the model mice daily administered Ritodrine (200 mg / kg i.p.) for 2 weeks and then measured the concentration of the biomarkers in serum. In addition, we employed DNA microarray analysis to interrogate gene expression levels in their liver.
Our microarray analysis showed that several genes related to the glucose and lipid metabolism were markedly up- or down-regulated in the liver of the Ritodrine-injected mice compared with controls. Indeed, the body weight in Ritodrine group had increased significantly and their fasting blood glucose levels were decreased. Our results suggest that β2-adrenergic signals possibly interfere with the appropriate glucose and lipid metabolism in liver and this metabolic abnormality may be one of the causes of the hepatic dysfunction.

P1L-A14-2 4-Oxononenal, but not 4-hydroxynonenal, converts xanthine dehydrogenase into oxidase in rat liver cytosol in vitro

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Xanthine oxidase /xanthine dehydrogenase (XO/XD) oxidizes oxypurines to uric acid, and only XO form producing reactive oxygen species. In the present study, the effects of 4-hydroxynonenal and 4-oxononenal on the conversion of XD to XO in rat liver were examined. The partially purified enzyme fraction from rat liver was incubated with xanthine in the presence or absence of NAD^+, and uric acid formed was measured by HPLC. Under basal conditions, XO activity represented only 15% of the total XO plus XD activity. 4-Hydroxynonenal up to 15 μM showed no significant effect on the activities of XO and XD, and their conversion. On the other hand, 4-oxononenal induced the conversion of XD to XO at concentrations ranging from 5 to 15 μM, and the concentration required for increasing XO activity by 50% was approximately 10 μM. These results suggest that 4-oxononenal, but not 4-hydroxynonenal, can be a stimulator of generation of reactive oxygen species through the conversion of XD to XO in liver.

P1L-A14-3 A simplified method to determine five cytochrome P450 (CYP) probe drugs by HPLC in a single run

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A simplified, rapid, selective and sensitive HPLC method for determining five cytochrome P450 (CYP) probe drugs in single run is described. The five specific probe substrates of caffeine, chlorzoxazone, tolbutamide, metoprolol and midazolam, together with the internal standard diazepam, were extracted using liquid-liquid extraction in rat plasma, followed by chromatography using a C_{18} column (5 μm particle size, 250 × 4.6 mm i.d.). The mobile phase consisted of a methanol and 50 mM phosphate buffer (pH 3.4, 65:35). All analytes were separated simultaneously in a single run that lasted less than 22 min. The detection limits range from 0.2~50 μg/ml for caffeine, 0.5~50 μg/ml for tolbutamide, metoprolol and midazolam, 0.2~100 μg/ml for chlorzoxazone, respectively. The intra- and inter-day precisions for five probe substrates were 1.38%~11.10% and 3.39%~11.33%, respectively, and the accuracy of five probe substrates ranged from 94.92%~113.06% and 92.18%~112.62%. The limit of quantification (LOQ) was 0.5 μg/ml for tolbutamide, midazolam and metoprolol, 0.2 μg/ml for caffeine and chlorzoxazone. The present method provides a robust, fast analytical tool for the five-probe drug cocktail. Finally, the method was suitable for determining the plasma concentration of these compounds and evaluating the CYP1A2, 2C9, 2D6, 2E1 and 3A4 activities in rats.

P1L-A14-4 A HPLC method for the determination of vancomycin in human serum

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Objective: To establish a HPLC method for the determination of vancomycin in human serum. Method: The vancomcin in serum extracted by the sedimentation with 70%-72% Perchlorate was determined through Agilent TC-C18 with 10% acetonitrile in potassium dihydrogen phosphate (0.05M). Other HPLC conditions were optimized as follows: the elution rate was 1mL·min^{-1}, the injection volume was 50 μL, the column temperature was 30°C and the detection wavelength was 236 nm. **Result:** Linearity was obtained from 1 to 80mg/L of vancomycin in serum. The recoveries were more than 70% (88%~110%, n=5) in the low-middle-high concentration. The intra-and inter-day RSD were less than 15% (1%-14%, n=5). **Conclusion:** we established the HPLC method of the determination of vancomycin in human serum. The method is rapid, simple and accurate.