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

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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.

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) deorphanizing 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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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.

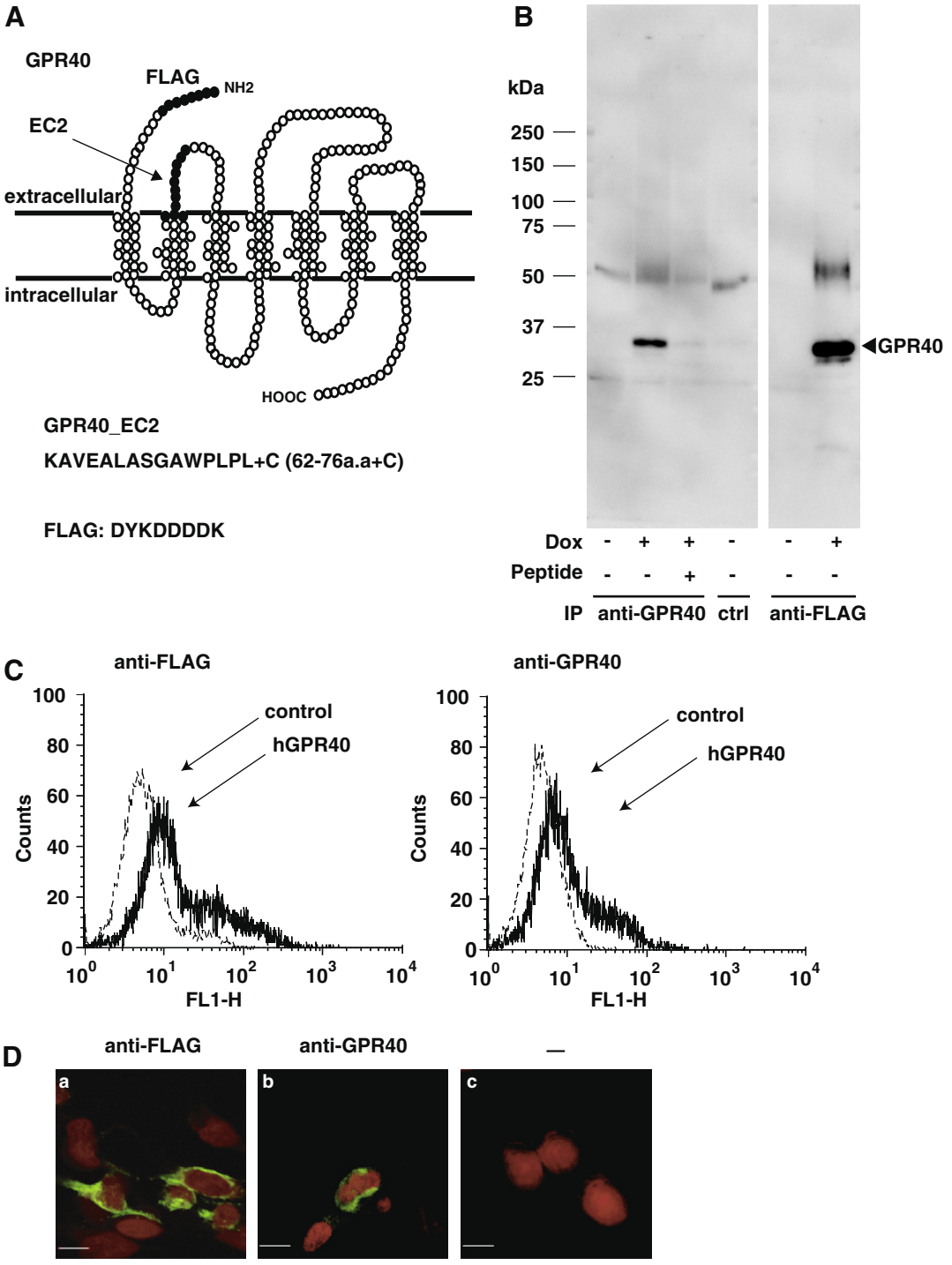

Fig. 1. Characterization of the anti-GPR40 monoclonal antibody in GPR40-transfected HEK cells. (A) Schematic diagram of GPR40. Following the generally accepted model of G protein-coupled receptors, the seven highly homologous hydrophobic regions are shown as membrane-spanning domains. The amino acid 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.)

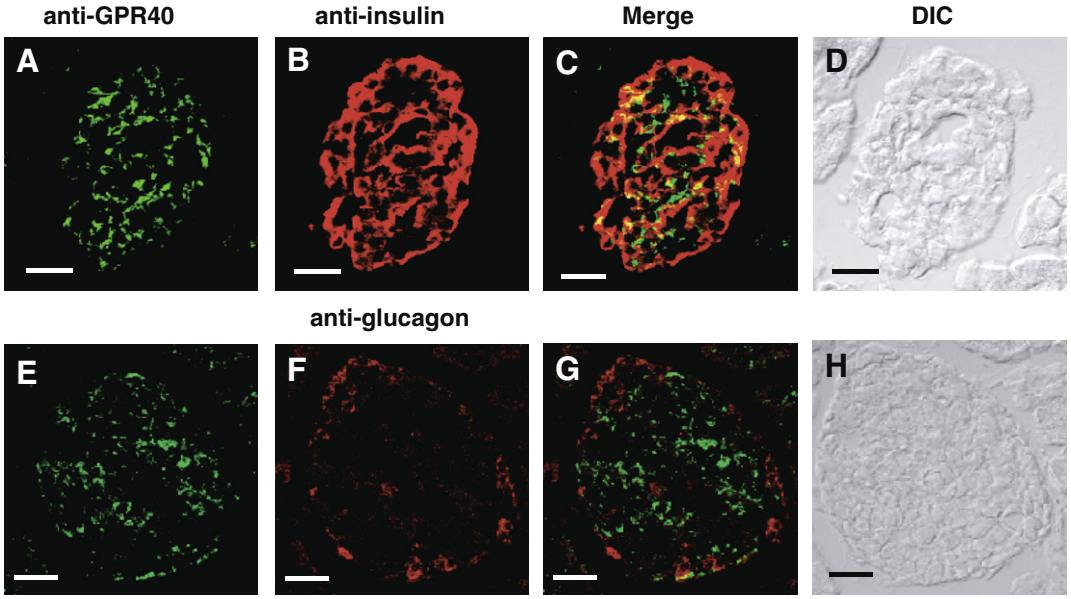

Fig. 2. Cross-staining of GPR40, insulin, and glucagon immunoreactivities. Confocal microscopy images of mouse pancreas, showing staining for GPR40 (A, E), insulin (B), glucagon (F), Merge (C, G), and DIC (D, H). Representative results from one of the three independent experiments are shown. Scale bar,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

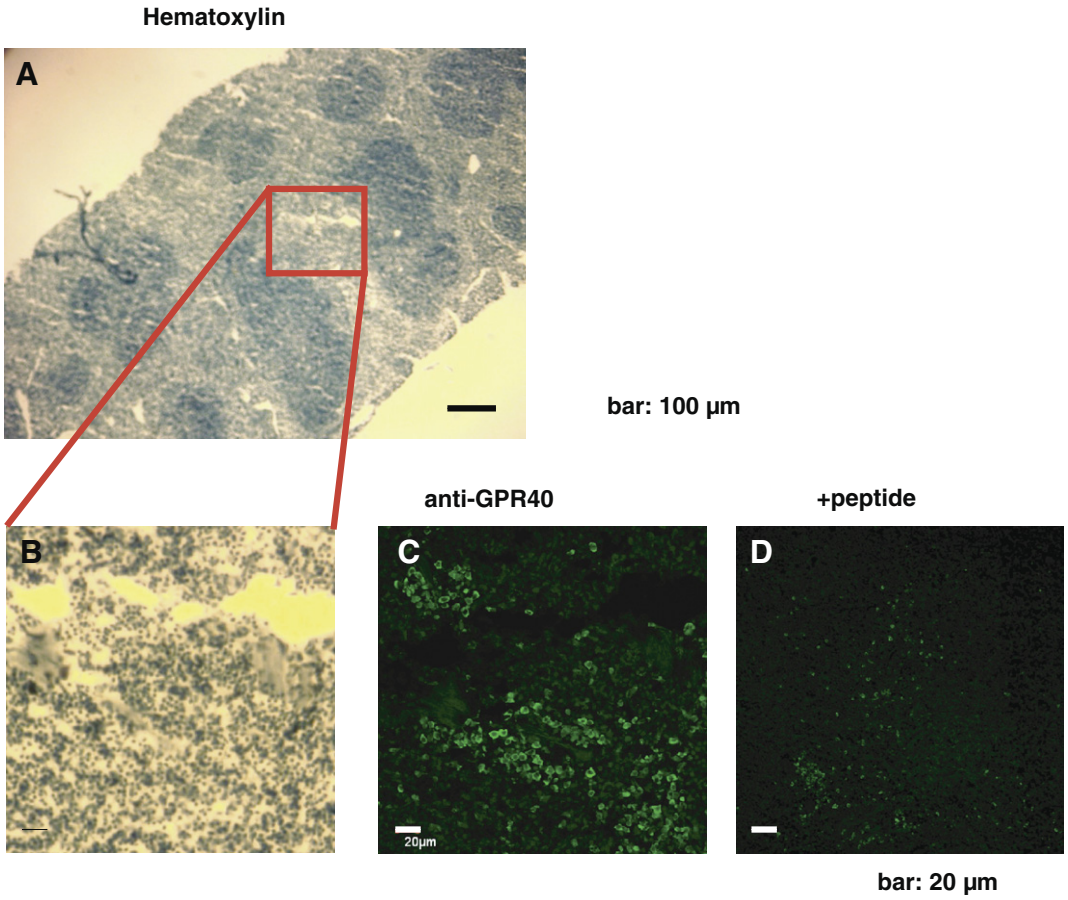

Fig. 3. Hematoxylin (A, B) and GPR40 immunohistochemical staining (C, D) in mouse spleen. For adsorption controls, the primary antibody was incubated with 10 μg/ml of the peptide used for immunizations. Representative results from one of the three independent experiments are shown. Scale bar, 20 μm.

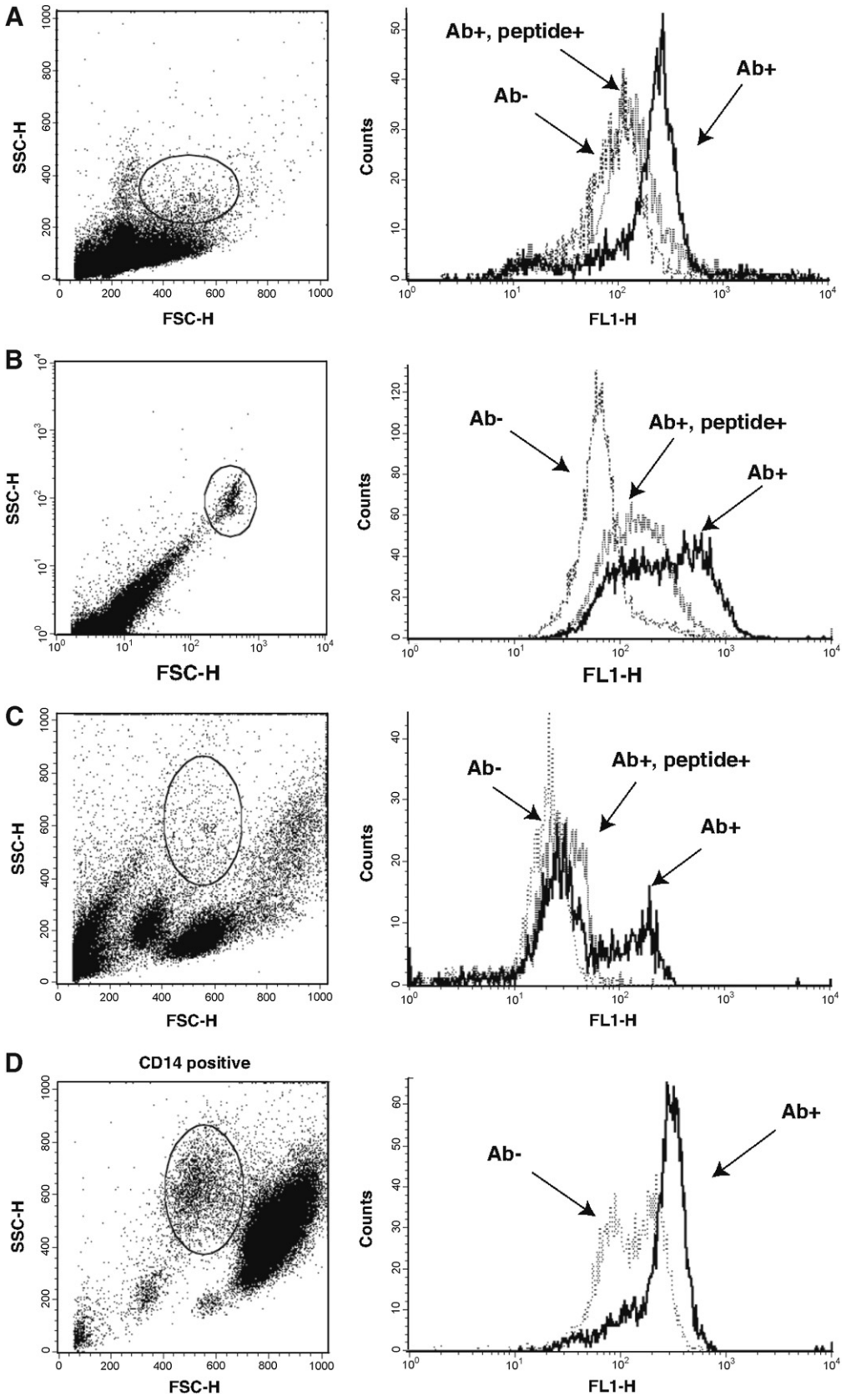

Fig. 4. Flow cytometry analysis of THP-1 cells and freshly isolated native cells. Freshly isolated mouse spleen cells (A), THP-1 cells (B), human PBMC (C), and human CD14-positive PBMC (D) were stained with the anti-GPR40 antibody (Ab+), the antibody with the immunogen peptide (+Peptide) or without the 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.

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