Development of an in vitro system for screening the ligands of a membrane glycoprotein CD36

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Abstract It has well been known that human and rodents exhibit a preference for fats. This suggests the existence of an orosensory system responsible for the detection of dietary fats. A plasma membrane glycoprotein CD36, besides the role in the uptake of long-chain fatty acids (LCFAs) as well as oxidized low-density lipoprotein (OxLDL) in a variety of cells, has been postulated to be a candidate fat taste receptor on the tongue. Therefore, molecules that bind with CD36 to cause intracellular signaling but have fewer calories could be substitutes for dietary fats. In the present study, we developed an in vitro system for the screening of CD36 ligands using Chinese hamster ovary-K1 cells (CHO-K1) stably transfected with human or mouse CD36. When incubated with OxLDL labeled with fluorescence dye, the fluorescence was much higher in the transfected CHO-K1 cells than in non-transfected CHO-K1 cells. Incubation of the transfected cells with OxLDL caused a rapid phosphorylation of extracellular signal regulated kinase,

and the degree was significantly higher compared with that in non-transfected CHO-K1 cells. The expression system using CHO-K1 cells could be a convenient tool to screen the novel ligands of CD36.

Keywords CD36 · Ligands · Oxidized LDL · Non-oxidized LDL · Binding · ERK1/2

Introduction

CD36 is an 88-kDa plasma membrane glycoprotein that functions as a scavenger receptor. This protein is expressed in a range of cells and tissues including platelets, monocytes/macrophages, vascular endothelial cells, and adipose tissues (Febbraio et al. 2001). CD36 recognizes a variety of ligands including collagen, thrombospondin-1 (TSP-1), long-chain fatty acids (LCFAs), oxidized low-density lipoprotein (OxLDL), anionic phospholipids, *Plasmodium falciparum*-infected erythrocytes and apoptotic cells (Abumrad et al. 1993; Febbraio et al. 2001; Greenwalt et al. 1992; Tandon et al. 1989). We previously reported that a fatty acid translocase, rat orthologue of CD36, was detected in the apical parts of the taste bud cells, possibly the gustatory cells in the circumvallate papillae (Fukuwatari et al. 1997). Furthermore, targeted disruption of CD36 gene strongly attenuated the preference for LCFA-enriched solutions and solid diets in mice (Laugerette et al. 2005). These findings

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suggest that CD36 is a receptor for LCFAs associated with the preference to the fats in the oral cavity. Fat- or fatty acid-related molecules that bind to CD36 and stimulate it to cause intracellular signaling but have fewer calories could be substitutes for dietary fats. In the present study, we developed an in vitro system for the screening of ligands of CD36 using CHO-K1 cells.

Materials and methods

Materials

Penicillin G (1,650 IU/mg), streptomycin sulfate (750 IU/mg), phosphate-buffered saline (PBS), RPMI1640 medium, Ham's F-12 medium, and polyclonal fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG antibody were purchased from Invitrogen (Carlsbad, CA, USA). Fatty acid-free bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO, USA). G418 was purchased from Wako Pure Chemicals (Tokyo, Japan). Polyclonal goat anti-human CD36 antibody (Cat. #AF1955) and Polyclonal goat anti-mouse CD36 antibody (Cat. #AF2519) were purchased from R&D Systems, Inc (Minneapolis, MN, USA). Monoclonal mouse anti-mouse CD36 antibody (Clone 63, Cat. #ABM5525) was purchased from Cascade Bioscience, Inc (Winchester, IN, USA). Monoclonal mouse anti-human CD36 antibody (FA6-152, Cat. #HM2122) was purchased from HyCult biotechnology B.V. (Uden, Netherlands). Polyclonal horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG antibody (Cat. #P0449) was purchased from Dako Japan (Tokyo). OxLDL was purchased from Biotium, Inc (Hayward, CA, USA). Non-oxidized LDL (nLDL) was purchased from Calbiochem (San Diego, CA, USA). Other chemicals were of the best grade available from commercial sources.

Cell culture and establishment of stable transformant

THP-1 cells were maintained at 37 °C in RPMI1640 medium supplemented with 100 units/ml penicillin and 100 units/ml streptomycin containing 10% fetal calf serum. CHO-K1 cells were maintained at 37 °C in Ham's F-12 medium supplemented with 100 units/ml

penicillin and 100 units/ml streptomycin containing 5% fetal calf serum. The cDNAs of human and mouse CD36 were amplified from human and mouse spleen cDNA libraries by polymerase chain reaction using the following primers, respectively: human; sense, 5'-ACCATGGGCTGTGACCGGAAC-3'; and anti-sense, 5'-GTTTATTTTATTGTTTTTCGATCTGCATGC-3' mouse; sense, 5'-ACCATGGGCTGTGATCGGAAGTGC-3'; and antisense, 5'-ACTTATTTTCCA TTCTTGGATTGCAAG-3'. The amplified human and mouse CD36 cDNAs were sequenced, subcloned into a mammalian expression vector, pcDNA3.1 (Invitrogen), and transfected into CHO-K1 cells using LipofectAMINE2000 (Invitrogen). To select clones expressing CD36, cells were cultured in Ham's F-12 medium supplemented with 100 units/ml penicillin, 100 units/ml streptomycin, and 0.5 mg/ml G418 containing 5% fetal calf serum. Positive clones were selected by means of Western Blotting with polyclonal goat anti-CD36 antibodies. CHO-K1 clones expressing human CD36 or mouse CD36 were designated as CHO-hCD36 or CHO-mCD36, respectively.

Western blotting analysis

Cells were washed with ice-cold PBS and lysed in SDS sample buffer and boiled for 5 min. Equal amounts of proteins were loaded on SDS-polyacrylamide gel electrophoresis (PAGE) (8% separating gel and 4.5% stacking gel). After electrophoresis, the proteins were transferred to PVDF membranes by electrotransfer. The PVDF membranes were blocked in PBS containing 0.1% Tween 20 (PBST) and 5% skim milk for 1 h at room temperature, and the washed three times with PBST. Then membranes were incubated overnight at 4 °C with the primary polyclonal goat anti-CD36 antibodies (Cat. #AF2519 for human CD36, #AF1955 for mouse CD36, 1:1,000 dilution) in PBST. Membranes were subsequently washed three times with PBS and incubated in the secondary HRP-conjugated anti-goat IgG antibody for 1 h at room temperature. Immunoreactive bands were detected by chemiluminescence.

Immunofluorescence

For immunofluorescence, cells (5×10^4) of the each transformant were seeded on 96-well plates, and

cultured for 24 h at 37 °C. Cells were washed with PBS, fixed with PBS 3% formaldehyde, and incubated for 20 min at room temperature. After several washing with PBS, the cells were incubated with PBS containing 2% BSA for 1 h at room temperature. After washing with PBS, the cells were incubated with the primary anti-human/mouse CD36 monoclonal antibody diluted in PBS containing 2% BSA for 1 h at room temperature (1:1,000). After the incubation, cells were washed three times with PBS and incubated with FITC-labeled polyclonal goat anti-mouse IgG antibody diluted in PBS containing 2% BSA for 1 h at room temperature (1:1,000). After several washing with PBS, cells were observed under confocal laser scanning microscope (CLSM) FLUOVIEW/LSM (Olympus, Tokyo, Japan).

Binding experiment

OxLDL was labeled using Alexa Fluor[®] 488 Protein Labeling Kit purchased from Invitrogen. After labeling, Alexa Fluor488-labeled-OxLDL (designated as AF488-OxLDL) was dialyzed against PBS containing 0.01% NaN₃. Cells (5×10^4) were seeded on 96-well plates, and cultured for 24 h at 37 °C before experiment. Cells were washed twice with PBS and exposed to PBS containing 100 µg/ml AF488-OxLDL in the presence or the absence of 10-fold excess of unlabeled ligand (OxLDL or nLDL), and incubated for 30 min at 37 °C. Cells were washed twice with PBS containing 0.01% BSA, exposed to PBS containing 3% formaldehyde, and incubated for 30 min at room temperature. After several washing with PBS, the fluorescence pattern in each well was imaged using CLSM. The fluorescence intensity in each image was calculated using Adobe photoshop[™] software (San Jose, CA, USA).

Extracellular signal regulated kinase (ERK1/2) activation assay

Cells (5×10^4) were seeded on 96-well plates and incubated in the medium for 24 h at 37 °C. After the incubation, each well was washed twice with PBS, exposed to PBS containing 0.1 mg/ml OxLDL or 0.1 mg/ml nLDL, and incubated for 10 min at 37 °C. The level of phosphorylated ERK1/2 (p-ERK1/2) was

determined using Cellular Activation of Signaling ELISA CASE[™] kit for ERK1/2 available from SuperArray Bioscience Corporation (Frederick, MD, USA). Briefly, the amount of p-ERK1/2 and cell number in each well were determined by reading absorbance at 450 and 595 nm, respectively, following the instruction of the manufacturer.

Results and discussion

Establishment of a stable transformant expressing human or mCD36

Of more than 20 single lines resistant to G418, a CHO-K1 line showing the strongest expression of human or mouse CD36 (CHO-hCD36 or CHO-mCD36) was selected based on the signal intensity on SDS-PAGE and Western blotting with polyclonal goat anti-human or mouse CD36 antibody, respectively. Both human and mouse CD36 proteins expressed in CHO-K1 cells migrated to the position corresponding to 88 kDa on SDS-PAGE (Fig. 1a). No apparent signals were found in the control parental CHO-K1 cells (Fig. 1a). The size of CD36 protein expressed in CHO-K1 cells differed from that in THP-1, a human monocyte cell line (Fig. 1a). This may be because of the differential glycosylation or the existence of alternative splicing variants for CD36 molecule in THP-1 cells. The cell surface localization of human or mouse CD36 was confirmed by immunofluorescence microscopy with anti-human or mouse CD36 monoclonal antibody, respectively (Fig. 1b).

Binding of OxLDL to CHO-hCD36 and CHO-mCD36

CLSM analysis revealed that AF488-OxLDL bound to CHO-hCD36 and CHO-mCD36, and 10-fold excess of unlabeled OxLDL greatly reduced the fluorescences (Fig. 2a). The binding of AF488-OxLDL to parental CHO-K1 cells was also shown. However, the fluorescence was much lower compared with that of CHO-hCD36 or CHO-mCD36 (Fig. 2a, b). The most likely explanation for the binding of AF488-OxLDL to parental CHO-K1 cells is that CHO-K1 cells endogenously express CD36 and/or receptor(s) for OxLDL

Fig. 1 Characterization of CD36 expressed by CHO-K1 cells. **(a)** Western blot analysis of cell lysates from parental CHO-K1 cells, CHO-hCD36, CHO-mCD36, and THP-1 cells. Experiments were performed as described in “Materials and methods.” Lane 1, parental CHO-K1 cells; lane 2, THP-1 cells; lane 3, CHO-hCD36; lane 4, parental CHO-K1 cells; lane 5, CHO-mCD36. **(b)** Immunofluorescence of CHO-hCD36 and CHO-mCD36. Experiments were performed as described in “Materials and methods”

such as LDL receptor (Brown and Goldstein 1979). Regardless, the difference in the fluorescence intensity between CHO-hCD36/mCD36 and parental CHO-K1 cells should correspond to the binding of OxLDL to CD36 expressed exogenously in the cells.

Binding of nLDL to CD36

It is controversial whether nLDL binds to CD36 (Boullier et al. 2000; Pearce et al. 1998). The fluorescence by the binding of AF488-OxLDL to CHO-hCD36 or CHO-mCD36 was greatly reduced in the presence of 10-fold excess of unlabeled nLDL (data not shown). This result suggests that nLDL as well as OxLDL can bind with CD36.

ERK1/2 activation

ERK1/2, a member of the MAP kinase superfamily, is known to participate in the signal transduction

Fig. 2 The binding of OxLDL to CD36 expressed by CHO-K1 cells. **(a)** Representative images obtained by CLSM. Parental CHO-K1 cells (a and b), CHO-hCD36 (c and d), and CHO-mCD36 (e and f) seeded on 96-well plates were incubated with 100 μ g/ml AF488-OxLDL for 30 min at 37 °C in the presence (b, d, and f) or absence (a, c, and e) of 10-fold excess of unlabeled OxLDL. **(b)** The fluorescence intensities in the images from parental CHO-K1 cells (CHO-K1), CHO-hCD36, and CHO-mCD36 incubated with 100 μ g/ml AF488-OxLDL for 30 min at 37 °C were determined as described in “Materials and methods.” Values are expressed as mean $\pm$ SEM ($n = 3$). * $p < 0.05$ (Student *t*-test)

Fig. 3 Increased ERK1/2 activation by the binding of OxLDL or nLDL to CD36 in the CHO-K1 cells stably transfected with CD36 cDNAs. The level of p-ERK1/2 and cell number in each well for parental CHO-K1 cells and the transfected CHO-K1 cells after stimulation by OxLDL or nLDL were determined as

described in “Materials and methods.” The amount of p-ERK1/2 (A₄₅₀) was normalized to the cell number (A₅₉₅). Values are expressed as mean ± SEM (*n* = 3). * *p* < 0.05 (Student *t*-test)

cascades controlling cell growth and differentiation (Hill and Treisman 1995; Marshall 1995) as well as cellular responses to cytokines and stress (Kyriakis and Avruch 2001; Tibbles and Woodgett 1999). It has previously been reported that signaling events induced by the binding of OxLDL to CD36 include the activation of ERK1/2 (Tabata et al. 2002). In the present study, we examined whether the binding of OxLDL or nLDL to the transfected CHO-K1 cells results in the activation of ERK1/2. For this purpose, the degree of ERK1/2 phosphorylation in the CHO-hCD36 or CHO-mCD36 was compared with that in parental CHO-K1 cells. Figure 3 shows that the amount of p-ERK1/2 after incubation with OxLDL or nLDL for 10 min was significantly higher in CHO-hCD36 or CHO-mCD36 than in parental CHO-K1 cells. This suggests that CHO-K1 cells possess essential components for the activation of ERK1/2 upon the binding of OxLDL or nLDL to CD36. Therefore, our system using the transfected CHO-K1 cells could be a convenient tool to screen the ligands of CD36.

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