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Lipid rafts regulate cellular CD40 receptor localization in vascular endothelial cells

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Abstract

Cholesterol enriched lipid rafts are considered to function as platforms involved in the regulation of membrane receptor signaling complex through the clustering of signaling molecules. In this study, we tested whether these specialized membrane microdomains affect CD40 localization in vitro and in vivo. Here, we provide evidence that upon CD40 ligand stimulation, endogenous and exogenous CD40 receptor is rapidly mobilized into lipid rafts compared with unstimulated HAECs. Efficient binding between CD40L and CD40 receptor also increases amounts of CD40 protein levels in lipid rafts. Deficiency of intracellular conserved C terminus of the CD40 cytoplasmic tail impairs CD40 partitioning in raft. Raft disorganization after methyl-β-cyclodextrin treatment diminishes CD40 localization into rafts. In vivo studies show that elevation of circulating cholesterol in high-cholesterol fed rabbits increases the cholesterol content and CD40 receptor localization in lipid rafts. These findings identify a physiological role for membrane lipid rafts as a critical regulator of CD40-mediated signal transduction and raise the possibility that certain pathologic conditions may be treated by altering CD40 signaling with drugs affecting its raft localization.

Keywords: CD40; Lipid rafts; Endothelial cells; Cholesterol

CD40 is 49 kDa integral membrane protein expressed on a variety of cells, including B-lymphocytes, monocytes, fibroblasts, epithelial, and endothelial cells, which shares significant sequence homology with the receptors for tumor necrosis factor receptor (TNFR) [1–3] CD40L, also referred as gp39 and recently renamed CD154, was first identified as a cell surface glycoprotein of 30–33 kDa and thought restricted to activated CD_4^+ T-lymphocytes [1–3] Recent reports have implicated both receptor and ligand closely correlated with several inflammatory diseases such as atherosclerosis [1–3].

Atherosclerosis morbidity has been linked to a Western diet, which includes high levels of red meat and saturated fat with higher cholesterol. Cholesterol, a neutral lipid that is a prominent component of the Western diet, contributes to the unique biophysical properties of the lipid raft microdomain. However, little is known on the relationship between lipid rafts and atherogenesis.

Membrane rafts are low density, detergent-insoluble membrane microdomains, enriched in sphingolipids, cholesterol, and glycosylphosphatidylinositol-linked proteins and found in all mammalian cell types [4]. Sphingolipids and cholesterol not only accumulate in detergent-resistant liquid-ordered lipid membrane microdomains, or membrane rafts, they are essential for raft formation [5,6]. These microdomains mechanistically contribute to signal transduction by raft proteins and play fundamental roles in diverse cellular functions, particularly in signal transduction [7,8]. Recently, several reports have demonstrated that CD40 recruitment to lipid rafts are thought to be intrinsic

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to some of the signaling functions of CD40 [9]. Several works, including ours, have highlighted the role of membrane rafts in the initiation of CD40 signal transduction and altered cholesterol distribution may influence CD40-induced inflammation [10,11].

Substantial evidence exists that phenotypic modulation of the endothelium to an activated state contributes to the pathogenesis of atherosclerosis. In this study, we aimed to assess whether lipid raft organization influenced the CD40 recruitment in vitro and in vivo.

Materials and methods

Cell culture. HAECs were obtained from Genentech, and maintained in endothelial basal medium supplemented with various growth factors and 2% fetal bovine serum (FBS).

Plasmid. Full-length wild-type human CD40 coding sequence was obtained by PCR and confirmed by sequencing. The primers used were: CGGGGTACCGCCACCATGG'ITCGTCTGCC TCTGCAG for the upstream primer and WI′GTCGACTCACTGT CTCTCCTGCAC for the downstream primer. The upstream primer had a built-in BamHI site and the downstream primer a SalI site (underlined) to facilitate cloning into the expression vector pCMV vector. Mutant CD40 receptor construct that lacked either highly conserved C terminus of CD40 (residues 261–289, Delta 260) and the extensive deletion of the cytoplasmic domain (STOP222) were made as previously described [12]. The expression constructs encoding CD40-GFP was constructed by Dr. Tao Yue (UT Southwestern Medical Center) and donated as gift. The cells (1×10^6) were plated per well in a six-well plate with 2 mL of medium. Cells were transfected once they had reached 80–90% confluence. The transfection efficiency was estimated by using GFP-plasmids.

Biochemical lipid raft separation. Rafts were isolated as described previously [13]. Briefly, postnuclear supernatant (PNS) from ECs (2×10^6) was solubilized in 1 mL buffer A [25 mmol/L Hepes (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 150 mmol/L NaCl, 1 mmol/L EGTA (ethyleneglycotetraacetic acid), protease inhibitors cocktail] containing 1% Brij 98 (Sigma–Aldrich) detergent for 5 min at 37 °C and was chilled on ice before it was placed at the bottom of a step sucrose gradient (1.33–0.9–0.867–0.833–0.8–0.767–0.733–0.7–0.6 mol/L sucrose). Gradients were centrifuged at 200,000*g* for 16 h in a Ti90 Beckman rotor (Beckman Instruments) at 4 °C. One-milliliter fractions were harvested from the top, except for the last fraction (no. 9), which contained 3 mL. The fraction contained the pooled fractions 1–4, and the heavy fraction (HF) consisted of pooled fractions 8 and 9.

For determination ganglioside GM1 content of density gradient fractions, 2.5×10^7 HAECs were incubated for 10 min at room temperature in 1 mL of culture medium containing 3.5 μg of HRP-labeled cholera toxin B subunit (or, as controls, untreated cells and cells incubated with an equivalent amount of unconjugated HRP). Cells were then subjected to lysis and density gradient centrifugation as described above. Gradient fractions were assayed for peroxidase activity by mixing 10 μL gradient fractions with 100 μL of 50 mM sodium phosphate, 25 mM citric acid (pH 5.0), 1 mg/mL *o*-phenylenediamine dihydrochloride, and 0.012% H_2O_2. Samples were incubated for 5 min at room temperature, and the reaction was stopped by adding 150 μL of 0.67 M sulfuric acid. Optical density of the samples was read at 405 nm in an enzyme-linked immunosorbent assay (ELISA) plate reader [14].

Immunoblotting. A total of 2×10^7 ECs in 1 mL medium were stimulated with 5 μg/mL of CD40 L for the indicated times at 37 °C, washed with cold PBS, lysed in 4 mL of MNX buffer [1% Triton X-100 in 25 mM MES, 150 mM NaCl (pH 6.5)] and subjected to lipid raft isolation. Soluble and lipid raft fractions were detected using immunoblotting with the specific antibody.

Confocal microscopy. Cellular surface CD40 receptor was detected using a recently described method [15], and lipid rafts were identified by the expression of the glycosphingolipid, GM1, which binds the B subunit of cholera toxin. Briefly, cells grown on glass coverslips were transfected with CD40-GFP construct, then incubated with CTxB-Alexa 488 (10 μg/mL) alone or in the presence of CD40L (5 μg/mL) for 1 h at 4 °C. Cells were fixed with 4% paraformaldehyde/PBS for 20 min and analyzed using a laser scanning confocal microscope (ZEISS).

Cholesterol depletion. For cholesterol depletion, 2.5×10^5 cells/mL was pretreated with 20 mM methyl-β-cyclodextrin (MβCD) for 30 min at 37 °C in serum-free medium. Cells were then washed three times with PBS and resuspended in complete culture medium [16].

Filipin staining. Cells were fixed in 3.2% PFA in PBS and then resuspended in PBS containing 30 μg/mL filipin, held at room temperature for 2 h, and directly analyzed on a flow cytometer [17].

Perfusion organ culture. Animal experiments were performed according to the guidelines of Sun Yat-Sen University for the care and use of laboratory animals which were approved by the Sun Yat-Sen University Animal Care Committee. Male New Zealand White rabbits (2–3 kg) were fed normal chow diet (control) or high-cholesterol diet (chow diet with 0.5% cholesterol) for 8 weeks. After treatment, the rabbits were anesthetized with ketamine (50 mg/kg IV) and xylazine (2 mg/kg IV) and arterial segments were isolated as previously described [18]. Organ culture of the aortic segments was carried out under sterile conditions in an incubator containing 5% CO_2 at 37 °C for 24–26 h.

Statistical analysis. Data are expressed as means ± SD. To compare the data between groups, ANOVA followed by post hoc statistical tests was used. A value of $P < 0.05$ was considered statistically significant.

Results

CD40 receptor associates with cholesterol-rich lipid rafts after membrane treatment with its ligand-CD40L

We initially assessed whether CD40 receptor localization in cholesterol-rich membrane lipid rafts during CD40 signaling by examining membrane localization of CD40 on HAECs using a biochemical approach [19]. As shown in Fig. 1A, none of CD40 receptor was found within lipid rafts, whereas CD40L-stimulation promoted the large majority of CD40 recruitment in this microdomain. As a control for proper separation, the membrane was probed with an antibody recognizing the raft marker protein Fyn, which is localized in rafts. As expected, Fyn appeared mainly in the light raft fractions, whereas the non-raft marker Rab5 was isolated in the heavy fractions (Fig. 1A).

A similar distribution pattern of CD40 receptor in the rafts after CD40L-stimulation was observed versus nonraft fraction (Fig. 1B). As a control, the raft marker flotilin was predominantly found in the raft fractions, whereas the cytoplasmic protein tubulin was exclusively detected in the nonraft fraction. These results indicated that CD40 receptor was associated with lipid rafts during CD40 signaling challenged with CD40L.

Next, the exogenous CD40 construct distribution in rafts was analyzed. We lysed HAECs transfected with CD40-GFP construct and fractionated the lysates by sucrose gradient centrifugation after CD40L-stimulation. Detection of GFP in lipid rafts indicated interaction of CD40 with cholesterol-rich membranes lipid rafts (Supplementary Fig. 1A). Lipid raft fractions were identified by the presence of the marker protein flotilin in light density factions 1, 2, 3, and 4 (Supplementary Fig. 1B). Confocal