Legends for supplementary Figures and Table

Supplementary Figure 1

Co-localization between LDLR and VEGFR1.

A. WCLs from 293T or RAW cells starved of serum for the indicated period of time 24 hours after transfection with VEGFR1-GFP were subjected to anti-LDLR and anti–actin immunoblotting.

B. 293T cells transiently transfected with VEGFR1-GFP were stimulated with 0 (-), 1, 10, and 100μ g/ml of nLDL. Mean numbers of endocytic vesicles larger than 0.35μ m in diameter that were calculated with 100 randomly selected cells were 5, 14, 42, and 42 per cell, respectively. VEGF (homodimer, 46kDa) 100ng/ml is equivalent to 6.37 μ g/ml nLDL (2930kDa)(Crouse et al, 1985) on a molar basis.

C. WCLs from CHO9 cells transfected with LDLR-FLAG vector or mock (-) were immunoblotted with anti-FLAG antibody.

D. CHO9 cells co-transfected with LDLR-FLAG (red) and VEGFR1-GFP (green) expression vectors were incubated with 100μ g/ml non-labeled nLDL in the presence or absence of SU5416, or 100ng/ml VEGF for 15 min.

LDLR-FLAG was visualized by immunostaining with anti-LDLR antibody. Note merged images with nLDL but not VEGF. Bars; 10μ m

Supplementary Figure 2

A. 293T cells transfected with LDLR-FLAG (green) (upper panels) or VEGFR1-GFP (green) (lower panels) were incubated with nLDL and immunostained with anti-clathrin (red) antibody. Bars; 10μ m

B. Upper panel: RAW cells transfected with the LDLR-FLAG expression vector were selected for G418 resistant clones with 400μ g/ml G418 for 2 weeks. RAW cells stably expressing LDLR-FLAG (RAW/LDLR-FLAG) were unstimulated (-) or stimulated (+) with nLDL. nLDL-stimulated internalization of LDLR-FLAG was visualized by immunostaining with anti-LDLR antibody.

Lower panel: WCLs (upper) or anti-FLAG IPs (lower) from RAW/LDLR-FLAG cells were immunoblotted with anti-FLAG (upper) or with anti-VEGFR1 antibody. VEGFR1 bound to a heparin column is shown (H, lower) as a size control.

C. Skin fibroblasts from WT and LDLR-/- mice were transfected with

VEGFR1-GFP and then stimulated with nLDL at 10μ g/ml.

Supplementary Figure 3

^{125}I-nLDL internalization assay

Preparation of LDL and LPDS and the internalization assay were performed basically as described by Goldstein et al (1983). LDL was isolated from plasma of healthy volunteers by sequential ultracentrifugation (density range of 1.019-1.063g/ml), and iodinated with Na^{125}I. 3T3/VEGFR1 cells cultured with LPDS (prepared also by ultracentrifugation) or 1% FCS for 24 hours were incubated with ^{125}I-LDL at 37℃ for 2 hours in the presence or absence of 50-fold excess of unlabeled LDL to determine specific LDL uptake. The cells were then chilled at 4℃, washed 5 times, and incubated with buffer containing dextran sulfate for 1 hour. After removal of dextran sulfate, the cells were lysed with 0.1N NaOH, and the radioactivity in the NaOH fraction that represents the amount of intracellular LDL was measured.

Supplementary Figure 4

nLDL induces protein degradation of VEGFR1.

A, B. WCLs from 3T3/VEGFR1 cells incubated with 500μ g/ml nLDL (A) or 100ng/ml VEGF (B) for the indicated time were immunoblotted (IB) with anti-LDLR, anti-VEGFR1, and anti-actin antibody. The experiments were repeated eight times and densitometric quantification is shown on the right.

Supplementary Figure 5

Hsp90 inhibitors induce VEGFR1 degradation

A-D. 3T3/VEGFR1 cells (A, D), NIH3T3 cells transiently transfected with VEGFR1-GFP (B) or RAW cells (C) were incubated with Hsp90 inhibitors for the indicated time. WCLs (A, D) or heparin-bound fraction (upper in C) were immunoblotted (IB) with anti-VEGFR1 antibody. The geldanamycin (GA)-induced internalization of VEGFR1 was visualized by GFP signals (B). Note that VEGFR1 was degraded (A, C) while LDLR remained undegraded (D).

Supplementary Figure 6

nLDL induces ubiquitination of VEGFR1

A. 3T3/VEGFR1 cells transfected with ubiquitin-FLAG expression vector were stimulated with mock (-), VEGF, GA, or nLDL. WCLs with or without the transfection, and anti-VEGFR1 IPs from each preparation were immunoblotted with anti-VEGFR1 (left) or anti-FLAG (right) antibody.

B. 3T3/VEGFR1 cells pre-incubated with mock (-), MG132 (MG), or Bafilomycin (Baf) were stimulated with 100ng/ml VEGF, 500μ g/ml nLDL, or 10μ M GA. WCLs were immunoblotted with anti-VEGFR1 and anti-actin antibody. Relative averaging values of the intensities of bands from three independent experiments are shown below the Western panels (A, C, E, F).

C. 3T3/VEGFR1 cells were stimulated by nLDL that was pre-treated with mock (-) or heparinase (+) at 1U/ml for 1 hour. WCLs were subjected to immunoblotting with anti-phosphotyrosine (PY), anti-VEGFR1 and anti-actin antibodies.

Supplementary Figure 7

Anti-MCP-1 antibody failed to block nLDL-induced cell migration

Three days after the intraperitoneal injection of thioglycolate solution,

peritoneal macrophages were isolated, washed with PBS and resuspended with 1% FCS containing medium at 2×10^6 cells/ml concentration. Approximately 100μ l of the cell suspension per well was loaded to the upper wells and incubated for 4 hours at 37℃. MCP-1 or nLDL at the indicated concentrations in the presence or absence of anti-MCP-1 blocking antibody (Ab) was applied. Data were corrected for background intensities with no stimulation in WT mice, and expressed as mean ±SD. *: $P<0.001$. **: $P<0.01$. Assays were performed in triplicate.

Supplementary Table 1

List of molecular reagents

Molecular reagents including antibodies, chemicals and transfection reagents, with applied cells in parentheses, are listed. Primers for the anti-LDLR siRNA experiment are also shown.