Probucol Pretreatment Enhances the Chemotaxis of Mouse Peritoneal Macrophages

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To investigate the effects of probucol on macrophage chemotaxis, we preincubated mouse peritoneal macrophages with probucol for 20 hours in vitro and using a modified Boyden chamber system compared their chemotactic responses with those of control macrophages that were preincubated with vehicle. Probucol pretreatment enhanced the macrophage chemotactic responses to zymosan-activated serum, acetylated low density lipoprotein (LDL), and native LDL. Probucol pretreatment also enhanced the basal migration observed when there was no stimulant in the lower chamber of a modified Boyden chamber. The chemotactic activity of native LDL was weaker than that of zymosan-activated serum in control macrophages; however, both substances became equally potent when the macrophages were preincubated with probucol. The degree of the enhancement to native LDL after probucol preincubation reached fourfold to eightfold. The fashion of the enhanced migration of macrophages to native LDL after preincubation with probucol was predominantly chemotactic rather than chemokinetic. Time-course experiments revealed that it took more than 12 hours of probucol preincubation to show clearly enhanced macrophage chemotaxis to native LDL. Macrophages preincubated with probucol together with cycloheximide showed markedly reduced chemotaxis compared with macrophages preincubated only with probucol. Probucol pretreatment also enhanced macrophage chemotactic responses to high density lipoprotein, oxidized LDL, and lipoprotein-deficient serum. On the other hand, probucol pretreatment did not enhance macrophage chemotaxis to simple liposome, cholesterol, and the pure chemoattractant C5a.

Activated macrophages induced by the intraperitoneal injection of lipopolysaccharide showed an enhanced chemotactic response compared with control macrophages. However, probucol pretreatment enhanced the chemotactic responses to zymosan-activated serum, probucol pretreatment also enhanced the chemotactic responses to native LDL after preincubation with probucol was predominantly chemotactic rather than chemokinetic. The enhancement reached fourfold to eightfold. The fashion of the enhanced migration of macrophages to native LDL after preincubation with probucol was predominantly chemotactic rather than chemokinetic. Time-course experiments revealed that it took more than 12 hours of probucol preincubation to show clearly enhanced macrophage chemotaxis to native LDL. Macrophages preincubated with probucol together with cycloheximide showed markedly reduced chemotaxis compared with macrophages preincubated only with probucol. Probucol pretreatment also enhanced macrophage chemotactic responses to high density lipoprotein, oxidized LDL, and lipoprotein-deficient serum. On the other hand, probucol pretreatment did not enhance macrophage chemotaxis to simple liposome, cholesterol, and the pure chemoattractant C5a.

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strated that smooth muscle cells were predominant as the origin of foam cells in atherosclerotic lesions of probucol-treated WHHL rabbits; on the other hand, macrophages were predominant in control (untreated) WHHL rabbits, suggesting that the antiatherogenic effects of probucol might relate to a decrease of the macrophage content in lesion areas.

Therefore, we questioned whether probucol affects macrophage moving activity as another possible direct effect on macrophages. We investigated the effects of probucol on the chemotaxis of mouse peritoneal macrophages in vitro using a modified Boyden chamber system.

Methods

Materials

Dulbecco’s modified Eagle’s medium (DMEM), Dulbecco’s phosphate-buffered saline (PBS), and Hank’s balanced salt solution (HBSS) were purchased from Nissui Pharmaceutical Co., Tokyo, Japan. l-Glutamine and streptomyacin-penicillin solutions were from Flow Laboratories, North Ryde, Australia. Fetal calf serum (FCS), obtained from Hyclone Laboratories, Inc., Logan, Utah, was inactivated at 56°C for 30 minutes before use. Plastic tubes and Petri dishes were purchased from Falcon, Oxford, Calif. Zymosan A, cycloheximide, human recombinant complement C5a, and phorbol 12-myristate 13-acetate (PMA) were from Sigma Chemical Co., St. Louis, Mo. Lipopolysaccharide W Escherichia coli 055:B5 (LPS) was obtained from DIFCO Laboratories, Detroit, Mich. Bovine serum albumin (BSA) was from Armour Pharmaceutical, Kankakee, III. HCO-50 (castor oil) was from Nikko Chemicals, Tokyo, Japan. Probucol [4,4’-(isopropylidenedithio)-£>/s(2,6-di-<butylphenol)] was kindly supplied by Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan.

Lipoproteins

Human LDL (d=1.019–1.063 g/ml), high density lipoprotein, (HDL3) (d=1.085–1.125 g/ml), HDL3 (d=1.125–1.21 g/ml), and lipoprotein-deficient serum (LPDS, d=1.215 g/ml) were prepared from the plasma of healthy human subjects and were isolated by differential ultracentrifugation. 17 Acetylated LDL was prepared by the repeated addition of acetic anhydride to LDL as described. 18 For the preparation of oxidized LDL, 5.0 mg protein of LDL was suspended in 2 ml PBS with Ca2+ and Mg2+ [PBS(+)] containing 5 μM CuSO4, and was incubated at 37°C for 24 hours. 19 After modification, each lipoprotein was dialyzed against PBS without Ca2+ and Mg2+ [PBS(−)] containing 0.24 mM EDTA.

Macrophages

Peritoneal macrophages were harvested from unstimulated female DDY mice weighing 25–30 g obtained from Shimizu Laboratories, Kyoto, Japan, in PBS(+) as described. 20,21 To obtain activated macrophages, the mice were injected with 30 μg LPS intraperitoneally 4 days before the harvest. 22 The peritoneal perfusate was collected by centrifugation at 400g at 4°C for 10 minutes. After washing once with PBS(+), the cells were suspended in the culture medium (DMEM containing 10% FCS by volume, 100 unit/ml penicillin, and 100 μg/ml streptomycin) at a density of 3x106 cell/ml. Aliquots (8 ml) of this suspension were dispersed onto plastic Petri dishes (100×20 mm), and the cells were cultured in humidified air containing 5% CO2 at 37°C for 2 hours. Then the cells were washed twice with 8 ml DMEM without serum to remove nonadherent cells, and further incubation (preincubation) was performed in DMEM containing 10% FCS with several experimental conditions. After preincubation, the cells were washed three times with 8 ml PBS(−) and then detached by incubation with 8 ml PBS(−) containing 5% FCS and 0.2% EDTA at 4°C for 30 minutes with some flushing. 23 Detached cells were washed with 20 ml PBS(+) three times at 4°C and used for the chemotaxis assay after being resuspended in HBSS containing 0.2% BSA (HBSS-BSA) at a concentration of 2.0x106 viable cells/ml. Cell viability was determined by trypan blue exclusion, and more than 90% of the cells were viable in all experimental conditions. After these procedures, almost all cells were able to attach to the plastic dish.

Preparation of Probucol Solution

To solubilize probucol in the culture medium we used HCO-50. First, probucol was solubilized with 10 volumes of ethanol, two volumes of HCO-50, and 88 volumes of distilled water under stirring. Then this solution was added to the culture medium at a concentration of 1%. Final concentrations of ethanol and HCO-50 in the culture medium were 0.1% and 0.02%, respectively. Chemotactic activities of macrophages preincubated with this vehicle were not different from those of macrophages preincubated with culture medium only. Control experiments were performed after preincubation of cells with this vehicle.

Chemotaxis Assay

The chemotaxis assay was performed in a 48-well modified Boyden micro chemotaxis chamber (Neuro Probe, Cabin John, Md.) using a polyvinylpyrrolidone-free polycarbonate membrane filter with 5-μm pores (Nucleopore Corp., Pleasanton, Calif.) as described. 24 The lower wells were filled with 27 μl HBSS-BSA or HBSS-BSA containing the agent to be assayed. The upper wells were filled with 50 μl of the macrophage suspension (1.0x105 cells/well) prepared previously. Assays were run in duplicate or triplicate wells. After incubation in humidified air containing 5% CO2 at 37°C for 2 hours (except for incubation time-course experiments), the membrane filter was removed and the nonmigrated cells were scraped from its upper side. The filter was fixed with methanol, stained with Diff-Quick (Green-cross, Osaka, Japan), and then mounted on a glass slide. Migrated cells were counted with a ×40 objective and a ×10 ocular containing a 10-mm2 counting grid. Five grid areas were counted per well and the values were summed.

We used 1% zymosan-activated serum (ZAS) in the lower chamber as the positive control. The ZAS was prepared by incubating 1 mg/ml zymosan A in rat serum for 1 hour at 37°C, and the supernatant obtained after centrifugation at 17,600g was used. 25
Hara et al
Probucol and Macrophage Chemotaxis

FIGURE 1. Bar graph showing effects of preincubation with probucol on mouse peritoneal macrophage chemotaxis to native low density lipoprotein (LDL), acetyl LDL, and zymosan-activated rat serum (ZAS). Macrophages were preincubated with vehicle (open bars), 30 μM probucol (hatched bars), or 60 μM probucol (filled bars) for 20 hours and detached as described in “Methods.” Upper well received macrophages (1.0×10⁵ cells/well), and lower well received 100 μg/ml native LDL, 100 μg/ml acetyl LDL, 1% ZAS, or Hanks’ balanced salt solution containing 0.2% bovine serum albumin (none). Chemotactic responses were assayed after 2 hours of incubation. Data represent mean±SD of six samples from two separate assays.

FIGURE 2. Plot showing time course of chemotactic responses to native low density lipoprotein (LDL) in mouse peritoneal macrophages preincubated with probucol. Macrophages were preincubated with vehicle (●) or 60 μM probucol (○) for 20 hours and detached as described in “Methods.” Upper well received macrophages (1.0×10⁵ cells/well), and lower well received native 100 μg/ml LDL or Hanks’ balanced salt solution containing 0.2% bovine serum albumin. Chemotactic responses were assayed after 1, 2, 3, 5, and 7 hours of incubation. Net chemotactic responses were determined by subtracting basal migration observed when there was no stimulant in lower chamber at each time point. Data represent mean±SD of six samples from two separate assays.

FIGURE 3. Plot showing dose-response relation of native low density lipoprotein (LDL) on chemotaxis of mouse peritoneal macrophages preincubated with probucol. Macrophages were preincubated with vehicle (●) or 60 μM probucol (○) for 20 hours and detached as described in “Methods.” Upper well received macrophages (1.0×10⁵ cells/well), and lower well received several concentrations of native LDL. Chemotactic responses were assayed after 2 hours of incubation. Data represent mean±SD of six samples from two separate assays.

Other

The protein content of the lipoproteins was determined by the method of Lowry et al. Chemotaxis data are presented as mean±SD.

Results

We measured macrophage chemotaxis after preincubation with or without probucol using native LDL, acetylated LDL, and ZAS as chemoattractants. Control macrophages, which were cultured with vehicle for 20 hours, showed a clear chemotactic response to 1% ZAS (Figure 1). Both acetylated LDL and native LDL attracted control macrophages, but the chemotactically active potency of native LDL was weak compared with ZAS (Figure 1). Twenty hours’ preincubation with probucol enhanced macrophage chemotactic responses to all three substances in a dose-dependent manner, and the basal migration observed when there was no stimulant in the lower chamber was also enhanced (Figure 1). Probucol itself at 30 and 60 μM failed to be a chemoattractant for control and probucol-pretreated macrophages, and the coexistence of 60 μM probucol in the chemotaxis assay did not alter the control macrophage chemotactic responses to native LDL and ZAS (data not shown). The chemotactic responses of probucol-pretreated macrophages to native LDL, acetylated LDL, and ZAS were not largely different (Figure 1).

Using native LDL as a chemoattractant best demonstrated the enhancement of the chemotactic potency by probucol. Therefore, we next characterized the chemotactic activity of native LDL for probucol-pretreated macrophages. Migration to native LDL was time dependent and reached a maximum at 2–5 hours’ incubation in both control and probucol-pretreated macrophages; at all times tested, marked enhancement by probucol pretreatment was observed (Figure 2). Maximal migration to ZAS was observed at around 5 hours’ incubation in both control and probucol-pretreated macrophages, and probucol pre-treatment enhanced macrophage migration at all times tested (data not shown). Native LDL was chemotactic for probucol-pretreated macrophages in a dose-dependent manner, and at all doses tested marked enhancement by probucol pretreatment was observed (Figure 3). To determine whether the enhanced macrophage migration activity to native LDL is directed cell movement, a modified checkerboard assay was performed. Figure 4 shows that...
native LDL has a primarily chemotactic with some chemokinetic activity for probucol-pretreated macrophages, and the migration of control macrophages to native LDL was also chemotactic. Time-course experiments revealed that it took more than 12 hours of probucol preincubation to show clearly enhanced macrophage chemotaxis (Figure 5).

Figure 6 shows the effect of cycloheximide on the chemotaxis enhanced by probucol pretreatment. Twenty hours' preincubation with cycloheximide did not show a marked effect on control macrophages' chemotactic responses. However, macrophages preincubated with probucol together with cycloheximide showed markedly reduced chemotactic responses to all chemoattractants compared with macrophages preincubated only with probucol, suggesting the involvement of protein synthesis by macrophages in this enhanced chemotactic response (Figure 6). Viability of cycloheximide-pretreated macrophages was not altered, as described in "Methods." The enhanced basal migration activity of probucol-pretreated macrophages was also reduced by cycloheximide.

Because pretreatment with probucol enhanced macrophage chemotaxis to both native and acetylated LDL, we tested other lipoproteins. When we used HDL₂, HDL₅, and even oxidized LDL as chemoattractants, enhanced macrophage chemotaxis was observed (Figure 7A). However, neither 90 μg/ml simple liposome made from dimyristoyl phosphatidylcholine nor 50 μg/ml cholesterol were potent chemoattractants in this particular experiment (data not shown). Probucol-pretreated macrophages also showed enhanced chemotactic responses to LPDS (Figure 7B).

Because ZAS contains lipoproteins and other serum factors, the enhanced chemotactic responses of probucol-pretreated macrophages to ZAS may depend on these lipoproteins or other serum factors. Because the major chemotactic factor in ZAS is known as complement fragment C5a, we tested the effect of human recombinant C5a. Pretreatment with probucol did not further enhance macrophage chemotaxis to C5a (Figure 8A). Time-course experiments revealed that maximal migration to C5a was observed at 2–5 hours' incubation in both control and probucol-pretreated macrophages, and no enhancement by probucol pretreatment was
FIGURE 8. Bar graph showing effects of preincubation with probucol on mouse peritoneal macrophage chemotaxis to human recombinant C5a and rat serum (RS). Macrophages were preincubated with vehicle (open bars) or 60 μM probucol (filled bars) for 20 hours and detached as described in "Methods." Upper well received macrophages (1.0×10^5 cells/well), and lower well received 100 μg/ml native LDL, HDL_2, HDL_3, oxidized LDL, or Hanks' balanced salt solution containing 0.2% bovine serum albumin (none) in panel A, 100 μg/ml native LDL, 100 μg/ml LPDS, 500 μg/ml LPDS, or none in panel B. Chemotactic responses were assayed after 2 hours of incubation. Data represent mean±SD of six samples from two separate assays.

We investigated the effects of probucol on the chemotactic responses of mouse peritoneal macrophages. The chemotactic responses of mouse peritoneal macrophages preincubated with probucol were compared with those of control macrophages preincubated with vehicle. First, we used 1% ZAS as a positive control chemottractant. Although acetylated LDL but not native LDL was reported to be a chemottractant for macrophages, we tested both. Control macrophages were clearly chemotactic to ZAS, and they were also chemotactic to both acetylated and native LDL; how-
Macrophages were detached as described in "Methods." Incubation. Data represent mean ±SD of six samples from two separate assays.

FIGURE 9. Bar graph comparing chemotactic responses in mouse peritoneal macrophages preincubated with probucol and responses in lipopolysaccharide (LPS)-induced activated macrophages. Resident macrophages were incubated with vehicle (open bars) or 60 μM probucol (filled bars) for 20 hours, and activated macrophages obtained by intraperitoneal injection of 30 μg/mouse LPS 4 days before harvesting (hatched bars) were incubated with vehicle for 20 hours. Macrophages were detached as described in "Methods." Upper well received macrophages (1.0 × 10⁶ cells/well), and lower well received 100 ng/ml native low density lipoprotein (LDL), 100 ng/ml human recombinant C5a, or Hanks' balanced salt solution containing 0.2% bovine serum albumin (none). Chemotactic responses were assayed after 2 hours of incubation. Data represent mean ±SD of six samples from two separate assays.

The chemotaxis of macrophages is considered as an inflammatory response, migrating toward inflammatory mediators. We clearly show that preincubation of probucol-pretreated macrophages with that of LPS-induced activated macrophages. Basal migration and the chemotactic response to C5a in activated macrophages were enhanced compared with control macrophages. However, activated macrophages did not show an enhanced chemotactic response to native LDL (Figure 9). This result clearly demonstrates that the chemotaxis of probucol-pretreated macrophages is different from that of LPS-induced activated macrophages. Further study is needed to elucidate the mechanisms underlying the alteration of macrophage chemotaxis by probucol pretreatment.

The chemotaxis of macrophages is considered as an inflammatory response, migrating toward inflammatory mediators. We clearly show that preincubation of macrophages with probucol enhances their chemotactic response. However, we do not think this alteration of macrophages by probucol pretreatment is an inflammatory change because of the following evidence. First, probucol pretreatment did not enhance the macrophage chemotactic response to C5a, a typical inflammatory mediator. Second, LPS-induced activated macrophages, typical inflammatory cells, did not show the enhanced chemotaxis to native LDL.

In relation to atherosclerosis, the chemotaxis of monocytes, a precursor of macrophages, is considered as the initial step for developing atherosclerosis followed by maturation to macrophages in the subendothelial space and foam cell formation. However, the
chemotaxis of these macrophages matured from monocytes is not well understood in relation to atherogenesis. According to pathological investigations, macrophage-derived foam cells in the subendothelial space could egress toward the bloodstream in several animal models for atherosclerosis. Our experimental data show that probucol-pretreated macrophages strongly migrate to several plasma factors. Thus, we speculate that probucol can stimulate macrophage egress toward the blood stream, and our speculation can explain the observation by O'Brien et al. that the atherosclerotic lesions of probucol-treated WHHL rabbits were poor in macrophage-derived foam cells. This unique action of probucol to stimulate macrophage chemotaxis may be a novel mechanism of this drug in preventing the development of or stimulating the regression of atherosclerosis.

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Hara et al  Probucol and Macrophage Chemotaxis 599
Probucol pretreatment enhances the chemotaxis of mouse peritoneal macrophages.
S Hara, Y Nagano, M Sasada and T Kita

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