

Effect of LDL concentration polarization on the uptake of LDL by human endothelial cells and smooth muscle cells co-cultured

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To substantiate our hypothesis that concentration polarization of low-density lipoprotein (LDL) plays an important role in the localization of atherogenesis, we investigated the effects of wall shear stress and water filtration rate (or perfusion pressure) on the luminal surface LDL concentration (c_w) and the LDL uptake by human vascular endothelial cells and smooth muscle cells co-cultured on a permeable membrane using a parallel-plate flow chamber technique and a flow cytometry method. The results indicated that the uptake of fluorescent labeled LDL (DiI-LDL) by the co-cultured cells was positively correlated with c_w in a non-linear fashion. When c_w was low, the uptake increased very sharply with increasing c_w . Then the increase became gradual and the uptake was seemingly leveled out when c_w reached beyond 160 $\mu\text{g/ml}$. The present study therefore has provided further experimental evidence that concentration polarization may occur in the arterial system and have a positive correlation with the uptake of LDLs by the arterial wall, which gives support to our hypothesis regarding the localization of atherogenesis.

Keywords atherosclerosis; concentration polarization; shear stress; filtration rate

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Introduction

It has been well documented that early event leading to the genesis of atherosclerosis is the accumulation of cholesterol and other lipids within the intima. These lipid deposits are believed to be derived primarily from plasma lipoproteins, particularly low-density lipoproteins (LDLs). Experimental results indicated that atherogenic

lipids may seep into the arterial wall by infiltrating through leaky endothelial cell junctions in regions of high endothelial cell turnover [1–2]. Previous theoretical and experimental studies [3–7] have revealed that because the endothelium of artery displays low permeability to plasma proteins, the filtration flow across artery wall may cause ‘concentration polarization’ of lipids, a well-known mass transport phenomenon, at the blood/wall interface with the lipids increasing in concentration from bulk value towards interface [8–11]. As the blood vessel wall is directly exposed to luminal surface lipid concentration, we have reasoned that it is the concentration of lipids at the blood/vessel wall interface and not the bulk concentration of lipids in blood that has a positive correlation with lipid infiltration into the arterial wall. Based on this reasoning, we hypothesized that the concentration of atherogenic lipids at luminal surface may vary according to its location in the circulation, even if the bulk concentration remains constant. In areas of disturbed blood flow (flow separation and recirculation) with a low wall shear rate, the luminal surface lipid concentration may be elevated, resulting in higher lipid infiltration in these areas and leading to the localization of atherogenesis [3].

To clarify the correlation of lipid infiltration with the luminal surface lipid concentration and substantiate our hypothesis, in the present study, using a parallel-plate flow chamber technique and the method of flow cytometry to measure the fluorescence intensity of fluorescent labeled LDL (DiI-LDL), we investigated the effects of wall shear stress and pressure (hence filtration rate) on the luminal surface LDL concentration and the LDL uptake by human vascular endothelial cells (huECs) and human smooth muscle cells (huSMCs) co-cultured on a permeable membrane [12]. The present study provides further evidence in supporting our hypothesis.

Materials and Methods

Materials

RPMI 1640 medium was purchased from Invitrogen Corporation (California, USA); Dulbecco Modified Eagle's Medium (DMEM), penicillin, and streptomycin were from Sigma Chemical (St. Louis, USA); fetal calf serum (FCS) was from TBD (Beijing, China); Millicell-CM culture inserts (PICM 03050) were from Millipore (Bedford, USA); and 1,1'-dioctadecyl-3,3,3',3'-tetramethyl indocarbocyanine (DiI) was from Biomedical Technologies Inc. (Stoughton, USA).

Preparation of LDLs and DiI-LDLs

LDLs were prepared and purified from fresh plasma obtained from healthy volunteers by gradient ultracentrifugation according to the method of Redgrave *et al.* [12]. Briefly, very LDLs (VLDLs) were first discarded after spinning plasma at 105,000 *g* for 18 h. Subsequently, LDLs (density 1.019–1.063 g/ml) were prepared from VLDLs-free plasma layered with 1.063 g/ml KBr solution and spun at 105,000 *g* for 24 h. Then the LDLs were filter and sterilized through a 0.2 μm filter and dialyzed extensively against 0.9% saline and 0.01% EDTA (ethylenediaminetetraacetic acid). The purity of the LDLs prepared was over 90% (LDL/total proteins) [13]. The LDLs were stored in sterile polystyrene tubes at 4°C before use; therefore, the level of oxidation of the LDLs was very low. Prior to each perfusion experiment, the purified LDLs were labeled with DiI following the procedure described by Li *et al.* [14]. The labeled LDLs were added to the DMEM medium that was used as the experimental perfusion solution. Then, the concentration of the labeled LDLs in the prepared perfusion solution was determined by measuring the protein concentration of the labeled LDLs with Bradford protein assay kit (Sigma, St. Louis, USA).

Preparation of huECs and huSMCs

The huECs and huSMCs were isolated from human umbilical cords. First, blood in the umbilical cord was rinsed with cold phosphate-buffered saline (PBS) buffer. After 20 ml of 0.125% trypsinase was injected into the vein of the cord, the cord was incubated at 37°C for 20 min. Then, huECs were collected by perfusion with RPMI 1640 medium. The collected ECs were centrifuged for 5 min at 89 *g* and suspended in RPMI 1640 containing 20% FCS. The huSMCs were acquired by explanting pieces of tissue individually in a cell culture

dish described by Yen *et al.* [15]. The huECs and huSMCs were cultured, respectively, in RPMI 1640 and DMEM with 20% FCS (*V/V*), penicillin (100 U/ml), and streptomycin (100 mg/l) in a humidified environment of 5% CO₂ and 95% air at 37°C.

huEC and huSMC co-culture

Co-cultured huECs and huSMCs were prepared following the procedure described by Niwa *et al.* [16]. In brief, huSMCs were seeded on a Millicell-CM membrane (PICM 03050; Millipore), which has an effective surface area of 4.2 cm² with pores of 0.4 μm in diameter at a concentration of 1.0×10^6 cells/cm² and cultured for several days at 37°C. After confirmation by phase-contrast microscopy that the entire surface of the membranes was covered with huSMCs, huECs were seeded directly over the huSMCs at a density of 1.0×10^6 cells/cm². In order for the cultured cells to recover their cellular functions such as LDL scavenger receptor activity and assure the huECs to form a confluent monolayer overlaying on top of the whole surface of the cultured huSMCs, the co-cultured cells were incubated at 37°C for 2 days before experiment. The culture medium was renewed every 3–4 days. Prior to the flow experiment, the co-culture preparations were examined carefully by the method described by Niwa *et al.* [16] to assure that the layer of smooth muscle cells was covered completely, without any leaky spot, by the endothelial cells. Briefly, for each batch of co-culture preparations, one of them was selected randomly and incubated for 2 h in a culture medium containing acetylated LDL labeled with fluorescent dye (DiI-Ac-LDL, 5 $\mu\text{g}/\text{ml}$), which is taken up specifically by ECs. Then, the confluence extent of the co-cultured ECs was observed by fluorescent microscopy. Only co-cultures from the same batch as the one whose surface was confirmed to be completely covered with ECs were used in the experiments. Using this confirmation method, it was assured that the thicknesses of the co-culture preparations were quite even and the mean value of the thickness was $\sim 7.5 \mu\text{m}$.

Experimental setup

Figure 1 shows a schematic drawing of the experimental perfusion system. It consist of a head-tank, a downstream collecting reservoir, a modified parallel-plate flow chamber with a height of 0.5 cm and a width of 35 cm, a peristaltic flow pump to circulate the perfusion fluid (the cell culture medium), and a blender of air and CO₂ with a constant temperature ($37 \pm 1^\circ\text{C}$). All the components of the perfusion system were connected using

perfusion solution was kept at 10 $\mu\text{g/ml}$ and the shear stress was set at 1.0 Pa, meanwhile the tested perfusion pressure was chosen at 10, 100, and 200 mmHg, respectively.

When measuring the effect of DiI-LDL concentration of the perfusion solution, the perfusion pressure and the shear stress were kept at 100 mmHg and 1.0 Pa, respectively. The DiI-LDL concentrations of the perfusion solution tested were 10, 50 and 100 $\mu\text{g/ml}$, respectively.

A standard procedure was followed throughout the entire experiment. After the prepared cell culture insert was installed into the flow system, the flow chamber was subjected to flow at a shear stress of 1.2 Pa for 24 h to precondition the huECs co-cultured with huSMCs. For each experiment, the height of the head-tank and the resistance of the outlet tubing were adjusted so that a chosen flow rate (therefore wall shear stress) through the flow chamber could be obtained while the pressure within the flow chamber was maintained at a desired value.

Measurement of DiI-LDL uptake

DiI-LDL uptake by the co-cultured huECs and huSMCs was measured by a flow cytometric analysis described previously [17–19]. Briefly, after 2 h of perfusion flow, the cell culture insert was disassembled from the flow chamber and washed with cold PBS three times. Then the cells on the membrane of the cell culture insert were lysed off from the insert membrane with 0.125% trypsinase. The cell lysates were centrifuged at 89 g for 5 min to remove cell debris and washed with cold PBS three times again. Each cell pellet was re-suspended in 0.5 ml PBS at a density of $\sim 1 \times 10^6$ cells/ml and kept on ice for flow cytometric analysis. DiI-LDL uptake by the co-cultured ECs and SMCs was decided by fluorescence intensity detected by a fluorescence activated cell sorter (FACStar^{plus}, Becton Dickinson, San Jose, USA), which was equipped with a 15 mW 488 nm air-cooled argon laser and two standard filters (DF 530/30 and DF 585/42). FACStar^{plus} research software (Becton Dickinson) was used to analyze the recorded data. The averaged DiI-LDL uptake by each cell was expressed as a mean fluorescence intensity (MFI) per cell as assessed in fluorescence windows (channel numbers).

Filtration rate measurement

During each experiment, the filtration rate across the wall of the cell culture insert was measured following the same procedure described by Deng *et al.* [20] with the help of the calibrated pipette installed on the filtration rate measurement cell, which had an inner diameter of

1 mm (**Fig. 1**). Briefly, the filtration rate measurement cell on the abluminal side of the co-culture insert was filled with the same fluid as the perfusate. A thin rubber gasket was used to prevent leakage of the solution from the measurement cell. The increase of the liquid volume (ΔV) in the measurement cell because of the filtration flow across the wall of the Millicell-CM membrane can be determined by the liquid volume increase in the capillary. Then the filtration rate was calculated by dividing ΔV by the surface area of the cell culture insert. During each experiment, the measurement of filtration rate was repeated five times. The measured filtration rate across the wall of the cell culture insert was the mean value over the entire membrane area of the insert.

Estimation of DiI-LDL concentration at the luminal surface of the cell culture insert

In order to reveal the correlation between DiI-LDL uptake by the co-cultured cells and c_w (the DiI-LDL concentration at the luminal surface of the cell culture insert), c_w has to be estimated for each experiment. To do so, at the completion of each experiment, a 31-gauge sampling needle attached to a microliter syringe (Hamilton Company, Reno, USA) was inserted vertically through a pre-opened hole on the glass window and a liquid sample at the solution/the cell culture insert boundary was retrieved through the sampling needle. The DiI-LDL concentration of the retrieved liquid sample was used as the representative of c_w value. In order to minimize the disturbance of the perfusion flow produced by the needle, the sample was retrieved as quickly as possible. Then the liquid sample was measured for fluorescence intensity by a spectrofluorometer with a LS-50B reflected-light fluorometer (Perkin-Elmer, Westport, USA) at excitation and emission wavelengths of 549 and 564 nm, respectively. The fluorescence intensity was then converted to the concentration of lipoproteins with a calibration curve of DiI-LDL.

Statistical analysis of experiment data

Experiments were repeated at least three times under the same flow conditions. Statistical analyses were performed using the Origin 7.0 software. The results were presented as mean \pm SD. Differences were considered significant if $P < 0.05$.

Results

Effect of shear stress on DiI-LDL uptake

In this set of experiments, DiI-LDL concentration of the perfusion solution was kept at 10 $\mu\text{g/ml}$ and the perfusion

pressure within the flow chamber was maintained at 100 mmHg, at which the measured filtration rate across the cell culture insert was $(44.9 \pm 5.1) \times 10^{-5}$ cm/s. **Figure 2** showed the effect of a laminar shear flow on DiI-LDL uptake by the co-cultured huECs and huSMCs. The experimental results showed that the averaged fluorescence intensity of experimental samples (hence, the averaged uptake of DiI-LDLs by each cell) was adversely correlated with shear stress. It first dropped sharply when shear stress increased from 0 to 1.0 Pa. After that, it decreased gradually with increasing shear stress, seemingly with a tendency of reaching a constant value.

Effect of water filtration rate on DiI-LDL uptake

Figure 3 shows the effect of water filtration rate (or perfusion solution) on DiI-LDL uptake by the co-cultured cells. In this set of experiments, the DiI-LDL concentration of the perfusion solution was kept at 10 µg/ml and the shear stress was set at 1.0 Pa. As evident from the figure, the averaged DiI-LDL uptake by each cell had a strong positive correlation with filtration rate (or perfusion pressure), indicating that filtration rate (or perfusion pressure) has a significant impact on the uptake of DiI-LDLs by the co-cultured cells.

Effect of DiI-LDL concentration of perfusion solution on DiI-LDL uptake

To study the effect of DiI-LDL concentration of the perfusion solution on DiI-LDL uptake by the co-cultured

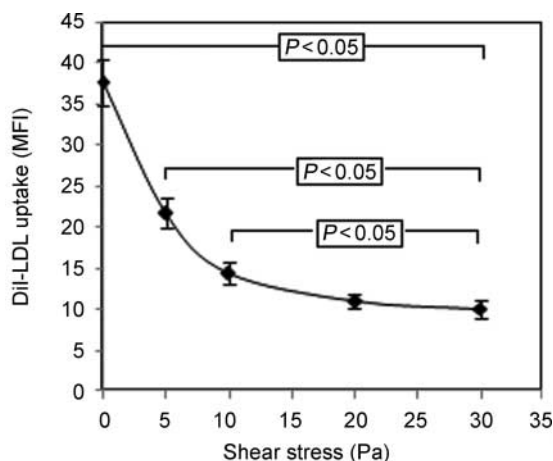


Fig. 2 Effect of shear stress on DiI-LDL uptake by the cultured cells expressed as the measured MFI per cell Shear stress was changed stepwise meanwhile the perfusion pressure and the DiI-LDL concentration of the perfusion solution were kept constant at 100 mmHg and 10 µg/ml, respectively. The results are expressed as mean ± SD (*n* = 3–5).

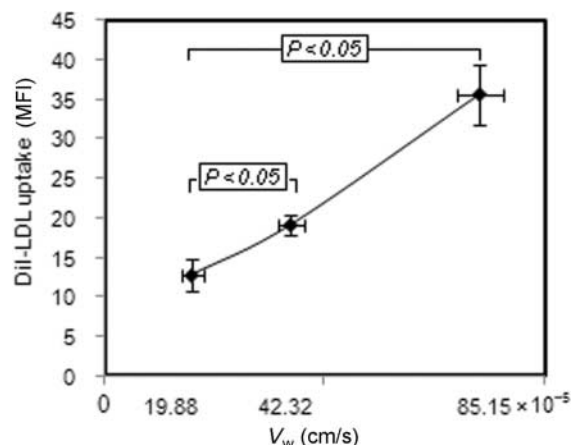


Fig. 3 Effect of water filtration rate (or perfusion pressure) on DiI-LDL uptake by the co-cultured cells expressed as the measured MFI per cell In this set of experiment, the water filtration rate was varied by changing the perfusion pressure stepwise (from 10 to 100, and to 200 mmHg). The results were expressed as mean ± SD. The horizontal bars are the standard deviations of the water filtration rates measured (*n* = 15–25), while the vertical bars are the standard deviations of DiI-LDL uptakes (*n* = 3–5).

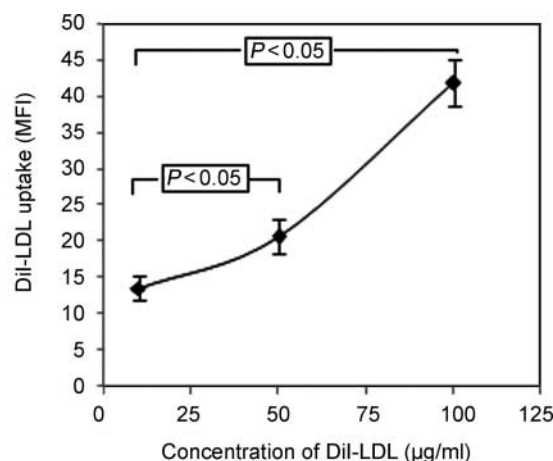


Fig. 4 Effect of DiI-LDL concentration on DiI-LDL uptake by the co-cultured cells expressed as the measured MFI per cell In this set of experiment, DiI-LDL concentration of the perfusion solution was changed stepwise meanwhile the perfusion pressure and the shear stress were kept constant at 100 mmHg and 1.0 Pa, respectively. The results are expressed as mean ± SD (*n* = 3–5).

cells, perfusion solutions with different concentration of DiI-LDLs (namely, 10, 50 and 100 µg/ml) were used in another set of experiments in which the perfusion pressure and the shear stress were kept at 100 mmHg and 1.0 Pa, respectively, at which the measured filtration rate across the cell culture insert was $(40.6 \pm 4.4) \times 10^{-5}$ cm/s. The results (**Fig. 4**) showed that the fluorescence intensity of the samples (hence, the averaged uptake of DiI-LDLs by

each co-cultured cell) increased with increasing DiI-LDL concentration of the perfusion solution.

Correlation between DiI-LDL uptake and the luminal surface concentration of DiI-LDLs

Figure 5 shows the correlation between DiI-LDL uptake by the co-cultured cells and the concentration of DiI-LDLs at the luminal surface of the cell culture insert. In Fig. 5, the luminal surface concentration of DiI-LDLs (c_w) for each experiment was determined as described previously. A regression analysis was performed for the data fitting as shown in Fig. 5. As evident from Fig. 5, the averaged DiI-LDL uptake by each co-cultured cell was positively correlated with the wall concentration of DiI-LDL. However, the correlation was highly non-linear. When c_w was low, the uptake increased very sharply with c_w . When c_w was higher than 70 $\mu\text{g/ml}$, the uptake increase became gradual with increasing c_w and the uptake- c_w curve would seemly reach a plateau beyond 160 $\mu\text{g/ml}$.

Discussion

The phenomenon of LDL concentration polarization has been predicted theoretically in the arterial system and verified experimentally *in vitro*. In recent years, more and more researchers have paid attention to this mass transport phenomenon and its possible role in the localization of atherogenesis [21–24]. In a cultured endothelial cell monolayer flow chamber system, Naiki *et al.* [4,7] studied the transport of macromolecules using 19 nm fluorescent microspheres as tracer particles. Their experiment

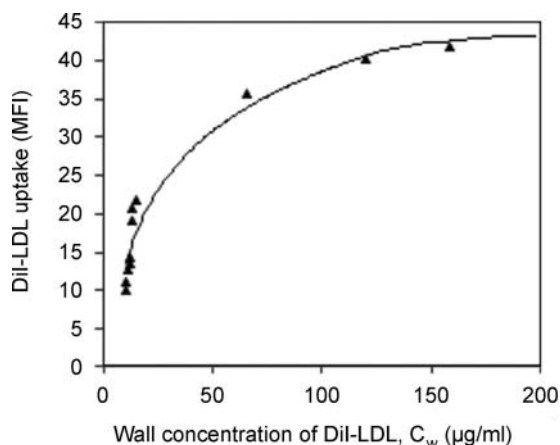


Fig. 5 Effect of the wall concentration of DiI-LDL, c_w , on DiI-LDL uptake by the co-cultured cells expressed as the measured MFI per cell. When c_w was low, the uptake increased very sharply with c_w , then the increase in the uptake became gradual. A plateau seemingly exists on the uptake- c_w curve.

demonstrated that flow-dependent concentration polarization of macromolecules occurred in the flow chamber at physiological wall shear rates and water filtration rates.

Experimental results by Wiklund *et al.* [25] suggest that the flux of LDLs into the arterial wall is not regulated by endothelial LDL receptors. Some cholesterol may seep into the arterial wall by infiltrating through leaky endothelial cell junctions. In the present study, possibly the measured DiI-LDL uptakes by the co-culture preparations were the results of DiI-LDL infiltration plus the receptor-mediated bindings of DiI-LDLs. Nevertheless, we believe that the majority of the measured uptakes were from the infiltration of DiI-LDLs into the preparations, which was not regulated by endothelial LDL receptors. This lipid infiltration should depend on the concentration of lipids at the blood/arterial wall interface as the arterial wall is directly exposed to the luminal surface lipid concentration. According to the theoretical analysis by Deng *et al.* [3], the distribution of atherogenic lipid concentration at the luminal surface (hence lipid infiltration into the arterial wall) is affected by local blood flow patterns. Using a spectrofluorometer technique, Naiki *et al.* [7] studied the effects of a shear flow on the uptake of LDL by a bovine aortic EC monoculture. Their results showed that the uptake of LDL by the cells increased with decreasing flow rate, indicating that flow-dependent concentration polarization of lipoproteins might indeed occur at the surface of the EC monolayer and affected the uptake of lipoproteins by the cultured ECs. However, their study did not reveal the direct correlation between LDL uptake and the luminal surface LDL concentration.

In the present study, we adopted a co-cultured huECs and huSMCs system that could be regarded as a physiological model of an arterial wall [16,26–29] and studied LDL uptake by the co-cultured cells. Due to the limitation of our experimental setup, it is impossible to distinguish the DiI-LDL uptake by the ECs from that of the SMCs. Therefore, a flow cytometry technique was utilized to measure the fluorescence intensity of DiI-LDLs uptaken by the co-cultured cells. The DiI-LDL uptake results were expressed as MFI per cell on the assumption that the total DiI-LDL uptake by the co-cultured ECs and SMCs on the cell culture insert tested is linearly correlated with MFI. Prior to each uptake experiment, we preconditioned the co-cultured huECs and huSMCs by subjecting the cells to shear flow at a shear stress of 1.2 Pa for 24 h. By so doing, we tried to make sure that the surface cells in all experiments are similar morphologically so that we can assume that the morphology of the surface cells is not a factor in the LDL uptake by the cells.

The results showed that the increase in perfusion pressure led to enhanced water filtration rate across the permeable membrane. This in turn resulted in elevated MFI of each cell measured (hence the averaged DiI-LDL uptake by each cell), indicating that the uptake of DiI-LDLs by the co-cultured cells increased with filtration rate. On the other hand, the flow-induced shear stress had an adverse effect on the uptake of DiI-LDLs by the co-cultured cells. It was also found that the DiI-LDL concentration of the perfusion solution had a positive impact on the DiI-LDL uptake by the co-cultured cells. Furthermore, the DiI-LDL concentration measurements of liquid samples retrieved at the solution/the cell culture insert boundary showed that the DiI-LDL wall concentration, c_w was always higher than the bulk concentration of DiI-LDL, c_0 , indicating that concentration polarization indeed occurred during all the flow experiments.

Previously, Deng *et al.* [3,6] showed that in an artery, due to the filtration flow across the vessel wall, the luminal surface macromolecule concentration c_w was always higher than the bulk concentration c_0 , and c_w increased almost linearly with filtration rate but decreased non-linearly with wall shear rate. When wall shear rate was low, c_w dropped very sharply with increasing wall shear rate. However, when wall shear rate increased further, c_w decreased with wall shear rate gradually with a tendency of approaching a constant value at certain level of wall shear rate. By comparing the present experimental study with the previous studies [3,6], it seems logic to conclude that the DiI-LDL uptake by the co-cultured cells was not directly affected by perfusion pressure and flow shear stress but was directly correlated with the luminal surface concentration of DiI-LDLs that was affected by perfusion pressure (filtration rate) and shear stress.

In order to clarify the correlation between the luminal surface concentration of DiI-LDLs and the DiI-LDL uptake by the co-cultured cells, we measured the c_w of DiI-LDLs for each experimental case, and plotted the uptake results against the c_w measured. The results showed that DiI-LDL uptake by the cells was positively correlated with c_w of DiI-LDLs in a non-linear fashion. When c_w was low, the uptake increased very sharply with increasing c_w . Then the increase became gradual and the uptake seemingly leveled out when c_w reached beyond 160 $\mu\text{g/ml}$. The explanation to account for this non-linear uptake of DiI-LDLs by the co-cultured cells is most likely as follows. In the present study, the measured MFI was the indication of DiI-LDL uptake by the co-cultured cells that possibly consisted of two parts: the receptor-mediated

bindings of DiI-LDLs and the DiI-LDL infiltration/accumulation. Before the receptors were saturated by LDLs, the measured uptake of DiI-LDLs was the result of the receptor-mediated bindings of DiI-LDLs plus DiI-LDL infiltration/accumulation. During this phase (i.e. when the wall concentration of DiI-LDLs, c_w , was low), the measured uptake of DiI-LDLs increased drastically with increasing c_w as shown in **Fig. 5**. After the receptors were saturated by the DiI-LDLs, the measured uptake was mainly due to the DiI-LDL infiltration/accumulation; therefore, the increase in DiI-LDL uptake became gradual (the second phase of the uptake curve in **Fig. 5**). Since in the co-cultured preparations the SMCs were not directly exposed to the perfusion solution, it is obvious that the DiI-LDL uptake by the SMCs was not directly affected by the luminal surface concentration of the DiI-LDLs but correlated with the DiI-LDLs infiltrated and accumulated within the co-cultured cell layers. It is possible that there exists a saturated concentration of DiI-LDLs within the co-cultured cell layers. This saturated concentration is the ceiling for the accumulation of the infiltrated DiI-LDLs within the cell layers. Therefore, when the saturated concentration within the cell layers was reached, the uptake stopped and the curve of DiI-LDL uptake was leveled out (the third phase of the curve in **Fig. 5**).

In summary, the present study has provided further experimental evidence that concentration polarization may occur in the arterial system and the uptake of LDLs by the arterial wall may be positively correlated with the luminal surface concentration of LDLs. These results therefore have given further support to our hypothesis regarding the localization of atherogenesis.

In the past years, many researchers have emphasized the importance of wall shear stress in atherogenesis. Although it has been widely recognized that the flow-induced shear stress plays a very important role in modulating endothelial functions and is one of the most important hemodynamic factors in vascular disorders such as atherosclerosis [30], it is most probable that the mass transport phenomenon of concentration polarization at the blood/arterial wall interface may also play an important role in the localization and development of atherosclerosis in the human circulation.

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