Upregulation of the Low Density Lipoprotein Receptor at the Blood–Brain Barrier: Intercommunications between Brain Capillary Endothelial Cells and Astrocytes

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Abstract. In contrast to the endothelial cells in large vessels where LDL receptors are downregulated, brain capillary endothelial cells in vivo express an LDL receptor. Using a cell culture model of the blood–brain barrier consisting of a coculture of brain capillary endothelial cells and astrocytes, we observed that the capacity of endothelial cells to bind LDL is enhanced threefold when cocultured with astrocytes. We next investigated the ability of astrocytes to modulate endothelial cell LDL receptor expression. We have shown that the lipid requirement of astrocytes increases the expression of endothelial cell LDL receptors. Experiments with dialysis membranes of different pore size showed that this effect is mediated by a soluble factor(s) with relative molecular mass somewhere between 3,500 and 14,000. Substituting astrocytes with smooth muscle cells or brain endothelium with endothelium from the aorta or the adrenal cortex did not enhance the luminal LDL receptor expression on endothelial cells, demonstrating the specificity of the interactions. This factor(s) is exclusively secreted by astrocytes cocultured with brain capillary endothelial cells, but it also upregulates the LDL receptor on other cell types. This study confirms the notion that the final fine tuning of cell differentiation is under local control.

C ELLS acquire cholesterol for membrane synthesis primarily by receptor-mediated uptake of LDL, which is internalized and delivered to lysosomes. LDL is then degraded and cholesterol is released to be used by the cells (Brown et al., 1981). These receptors appear to be regulated by a feedback mechanism responsive to the availability of LDL in the surrounding medium. These authors have suggested that LDL suppresses the synthesis of new receptor sites. In vivo, normal circulating concentration of lipoproteins downregulates the LDL receptor levels on vascular endothelial cells (ECs).[1]

Studies in situ indicate that the endothelial cells of the major vessel are highly contact inhibited, and that cell division takes place slowly, if at all, except in response to endothelial injury. Contact inhibition also leads to downregulation of LDL receptors.

A number of different studies have confirmed that growing ECs in culture also bind LDL (Vlodavsky et al., 1978). Kenagy et al. (1984) have found that the decreased ability of the LDL receptor of confluent ECs to degrade LDL is the result of the loss of LDL receptor activity. This decrease was also observed with fibroblasts and smooth muscle cells when they were cultured in complete medium containing serum and at confluence.

In contrast to what was observed with large vessels in vivo (Vasile et al., 1983) and in culture (Kenagy et al., 1984), we have shown (Méresse et al., 1989b) that confluent brain capillary ECs express an LDL receptor in vivo. This receptor exhibits the same characteristic properties as the LDL receptor on human fibroblasts (Goldstein and Brown, 1977). In addition, we have shown that cultured brain capillary ECs have LDL receptors with the same apparent molecular weight as in vivo (Méresse et al., 1991). How can we explain the difference between brain capillary ECs and peripheral ECs? ECs in different locations exhibit varying properties well suited to functional interactions between the blood and the underlying tissues (Fishman, 1982; Zetter, 1984). Brain capillary ECs, which constitute the blood–brain barrier, are sealed together by high electrical resistance tight junctions, and they display a low rate of transcellular vesicular transport (Reese and Karnovsky, 1967). These barrier properties contribute to the maintenance of the homeostasis of brain interstitial fluid (Brightman, 1989). In recent years, the manner in which capillary ECs in the brain become different from those in the periphery has been examined and the crucial role of the environment in which they grow has been

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1. *Abbreviations used in this paper*: ABAE, aortic bovine arch endothelial cells; ACE, adrenal cortex endothelial cells; AUF, astrocyte-regulating LDL receptor factor; BCECs, brain capillary endothelial cells; CS, calf serum; ECs, endothelial cells; GFAP, glial fibrillary acidic protein; LPDS, lipoprotein-deficient serum; SMC, smooth muscle cells.

465

demonstrated (Stewart and Wiley, 1981). Astrocytes, the nearest neighbor of brain capillaries, have been shown to induce some of the specialized properties of ECs (Janzer and Raff, 1987). In attempts to investigate such interactions, we have developed an in vitro model of the blood–brain barrier that imitates an in vivo situation by culturing brain capillary ECs and astrocytes on opposite sides of a filter (Dehouck et al., 1990a, 1992).

To understand why brain capillary ECs express LDL receptors in spite of the tight apposition of ECs and their contact with high concentrations of lipoproteins, we have investigated the capacity of astrocytes to modulate the expression of the LDL receptor on brain capillary ECs. We document specific EC–astrocyte interactions, and we propose that a soluble factor(s) derived from astrocytes modulates LDL receptor expression at the luminal surface of the brain capillary endothelium.

Materials and Methods

Cell Culture

Bovine Brain Capillary ECs. Brain capillary ECs were isolated and characterized as described by Méresse et al. (1989a). The use of cloned endothelial cells enables us to obtain a pure endothelial cell population without contamination by pericytes. The cells were cultured in the presence of DME supplemented with 15% (vol/vol) heat-inactivated calf serum (Hyclone Laboratories, Logan, UT), 2 mM glutamine, 50 μg/ml gentamycin, and basic fibroblast growth factor (1 ng/ml added every other day).

Bovine Aortic ECs. Bovine aortic ECs were isolated and characterized as described by Dehouck et al. (1990b).

Bovine Adrenal Cortex Endothelial Cells (ACE). ACE were isolated and characterized as described by Gospodarowicz et al. (1986). ACE (a gift from Dr. S. Saule, Molecular Oncology Laboratory, Institut Pasteur, Lille, France) were cultured as the bovine brain capillary endothelial cells were.

Rat Astrocytes. Primary cultures of mixed astrocytes were made from newborn rat cerebral cortex. After the meninges had been removed, the brain tissue was gently forced through a nylon sieve, as described by Booher and Sensenbrenner (1972). Astrocytes were plated on six multiwell dishes (Nunclon; Nunc A/S, Roskilde, Denmark) at a concentration of 1.2 × 10⁵ cells/ml in 2 ml of DME supplemented with 10% FCS (Hyclone Laboratories), and the medium was changed twice a week. 3 wk after seeding, cultures of astrocytes stabilized and were used for experiments. The astrocytes were characterized with glial fibrillary acidic protein (GFAP), and >95% of the population was GFAP positive (Dehouck et al., 1990b).

Coculture of Endothelial Cells and Astrocytes. Filters were prepared for coculture as follows: Culture plate inserts (Millicell-CM 0.4 μm, 30-mm diameter; Millipore Corp., Bedford, MA) were coated on the upper side with rat tail collagen prepared by a modification of the method of Bornstein (1958).

Experimental methods proceeded as follows: Cultures of astrocytes were prepared as described above. After 3 wk, coated filters were set in six multiwell dishes containing astrocytes, and ECs were plated on the upperside of the filters in 1.5 ml of medium with a concentration of 4 × 10⁵ cells/ml for bovine brain capillary ECs and bovine adrenal cortex ECs. Bovine aortic ECs were plated at the concentration of 2 × 10⁴ cells/ml. The medium used for the coculture was DME supplemented with 15% calf serum (CS), 2 mM glutamine, 50 μg/ml gentamycin, and 1 ng/ml basic fibroblast growth factor added every other day. This medium was changed every other day. Under these conditions, ECs form a confluent monolayer after 7 d. Experiments were performed 5 d after confluence. This arrangement readily permits the use of different cell types, which were separated easily after coculture by removing the insert.

Bovine Aortic Smooth Muscle Cells (SMC). SMC were isolated and characterized as described by Dehouck et al. (1990b).

Preparation of LDL, Acetylated LDL, and Lipoprotein-deficient Serum

LDL was isolated from human plasma by sequential ultracentrifugation at the densities of 1.03–1.053. The densities were adjusted using solid KBr. The LDL was extensively dialyzed at 4°C against 0.15 M NaCl. Acetylated LDL was prepared by treating LDL with acetic anhydride (Basu et al., 1976). LDL was radioiodinated as described by Bilheimer et al. (1972). After labeling, the LDL was chromatographed on a PD10 column (Pharmacia Fine Chemicals, Piscataway, NJ) and extensively dialyzed at 4°C against 0.15 M NaCl and 0.01% EDTA (pH 7.4). The specific activity of [¹²⁵I]-LDL, which was used within 10 d of preparation, ranged from 300 to 400 cpm/ng protein. Lipoprotein-deficient serum (LPDS) was prepared from FCS by ultracentrifugation at the density of 1.25 adjusted with solid KBr. LPDS was extensively dialyzed at 4°C against 0.15 M NaCl.

[¹²⁵I]LDL Binding

Cells were incubated for 2 h at 4°C with DME, 10 mM Hepes, 0.2% BSA, and the indicated concentrations of [¹²⁵I]LDL. At the end of the incubation, the cells were washed nine times at 4°C: three times with 4 ml of PBS (Ca⁺⁺/Mg⁺⁺), three times with 4 ml of PBS (Ca⁺⁺/Mg⁺⁺) containing 0.2% BSA, three times with 4 ml of PBS (Ca⁺⁺/Mg⁺⁺). Then, endothelial cell associated radioactivity was determined by removing the membrane of the culture insert and counting it in a gamma counter. Astrocytes cultured on the plastic were solubilized in 1 ml of 0.1 N NaOH, and aliquots were taken for measurements of radioactivity and protein concentrations. Nonspecific binding was determined by incubating the cells with [¹²⁵I]LDL and a 20-fold excess of unlabeled LDL. The same protocol was followed for acetylated LDL binding.

Inhibition of cell surface binding of [¹²⁵I]LDL to brain capillary ECs at 4°C by the monoclonal antibody, designated immunoglobulin C-7 (American Type Culture Collection, Rockville, MD) (Beisiegel et al., 1981) was achieved by incubating it in the same medium as the ECs at a concentration of 1 mg/ml. IgG directed against apolipoprotein AI at the same concentration was used as a control.

Studies of the Regulation of LDL Receptor

The induction of LDL receptor expression can be described in four phases (Fig. 1):

Coculture Phase. Brain capillary ECs and astrocytes were cocultured for 12 d in DME supplemented with 15% CS as described above.
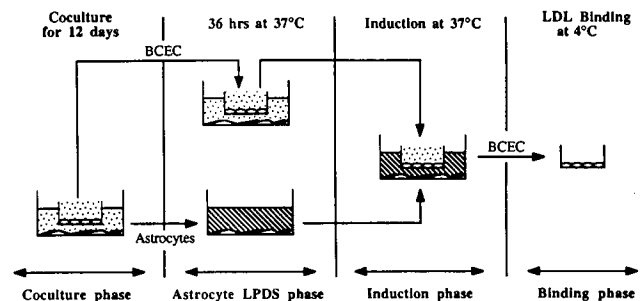

Figure 1. Experimental procedure for the upregulation experiments. The induction of LDL receptor expression can be described in four phases: (*Coculture phase*) Brain capillary ECs and astrocytes were cocultured for 12 d in DME supplemented with 15% CS. (*Astrocyte LPDS phase*) ECs were transferred and cocultured with other astrocytes in DME supplemented with 15% CS. Astrocytes of the initial coculture were incubated for 36 h at 37°C with DME supplemented with 10% LPDS, 2 mM glutamine, and 50 μg/ml gentamycin. As a control, the incubation of astrocytes was performed with DME supplemented with 10% FCS. (*Induction phase*) On the day of the experiment, brain capillary ECs in DMEM supplemented with 15% CS were cocultured for different times at 37°C with cholesterol-depleted astrocytes in their 36-h LPDS incubating medium. (*LDL Binding phase*) After the induction phase, specific [¹²⁵I]LDL binding to ECs was performed at 4°C, at the concentration of 45 μg/ml of [¹²⁵I]LDL as described under the experimental procedures. ▨, DME + 15% CS; ◩, DME + 10% LPDS;∞∞, brain capillary endothelial cells (BCECs); ▲▲, astrocytes.

Astrocyte LPDS Phase. ECs were transferred and cocultured with other astrocytes in fresh DME supplemented with 15% CS. Astrocytes of the original coculture were incubated for 36 h at 37°C with DME supplemented with 10% LPDS, 2 mM glutamine, and 50 μg/ml gentamycin. As a control, astrocytes were incubated with DME supplemented with 10% FCS.

Induction phase. On the day of the experiment, the filter inserts with ECs, in 1.5 ml of fresh DME supplemented with 15% CS, were moved to a well that contained astrocytes in their 36-h LPDS-conditioned medium. The cells were incubated together for different times at 37°C.

LDL Binding Phase. After the "induction phase," specific [^{125}I]LDL or ^{125}I-acetylated LDL bindings to ECs were performed at 4°C as described above. The induction phase was modified in two experiments: (*a*) brain capillary ECs were separated from cocultured astrocytes by a dialysis bag with a cut-off molecular weight of either 3,500 or 14,000 (see Fig. 7); (*b*) luminal medium contained 10 μg/ml cycloheximide (Sigma Immunochemicals, St. Louis, MO). In some experiments, the same protocol was followed, but bovine aortic ECs or bovine adrenal cortex ECs were plated instead of brain capillary ECs or SMC instead of astrocytes.

Cholesterol Concentration of Astrocytes

The concentration of cholesterol in astrocytes was determined by high pressure liquid chromatography using a reversed phase C-18 column as described by Barbaras et al. (1986).

Results

Coculture of Endothelial Cells and Astrocytes

Fig. 2 *a* illustrates the structure of confluent bovine brain capillary ECs cultured on an insert coated with collagen. ECs form a monolayer of small, tightly packed, nonoverlapping and contact-inhibited cells. This culture is a pure endothelial cell population without contamination by pericytes. The astrocytes were characterized with GFAP. As shown in Fig. 2 *b*, ~95% of the cell bodies and the processes of the astrocytes were stained.

Binding of LDL to Brain Capillary ECs

Labeled LDL bound with saturation kinetic on brain capillary ECs cultured alone or cocultured with astrocytes in 15% CS, in contrast to what was observed on aortic endothelial cells (Fig. 3). By Scatchard analysis, the data indicate the presence of a single binding site with an apparent K_d of 90 nM for ECs cultured alone and 43 nM for ECs cocultured
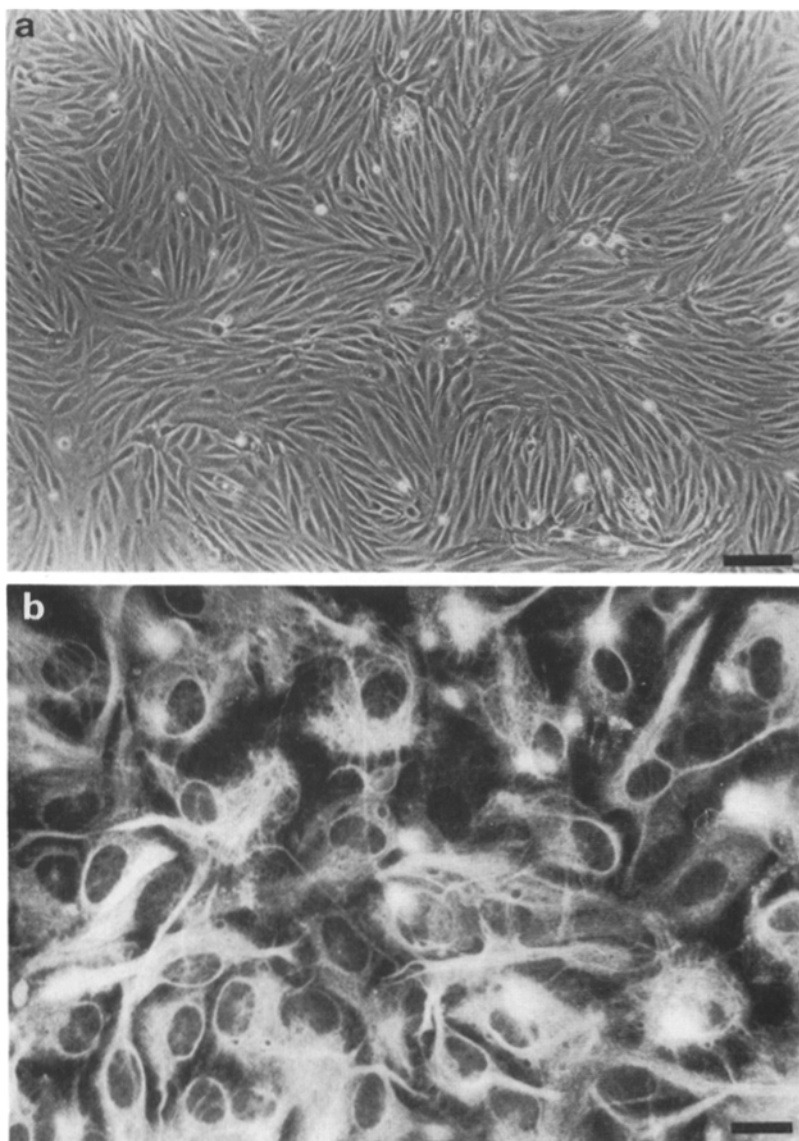

Figure 2. (*a*) Phase contrast micrograph of confluent brain capillary endothelial cells grown on the upper face of a collagen-coated filter. (*b*) Immunolabeling of a coculture of astrocytes with the anti-GFAP serum. Bars, 30 μm.
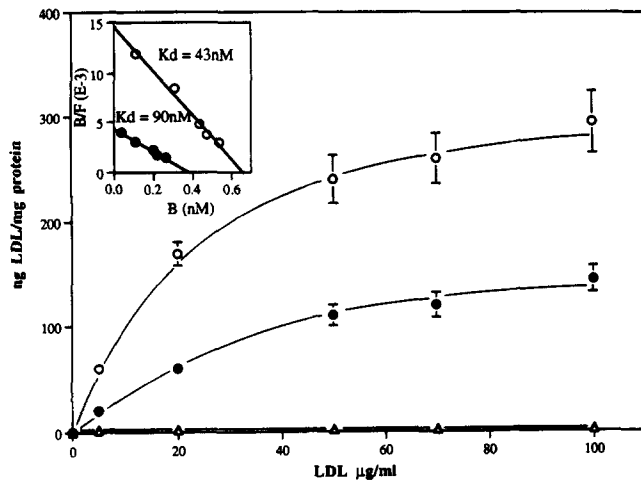
Figure 3. LDL binding to endothelial cells. Brain capillary ECs were cultured in the presence (○) or in the absence (●) of astrocytes for 12 d in DME supplemented with 15% CS. LDL binding was performed on ECs at 4°C as described under the experimental procedures. These data are compared with LDL binding on confluent aortic ECs (△). (Inset) Scatchard analysis of the binding data to brain capillary ECs. B, bound; B/F, bound/free.

with astrocytes. However, the maximal binding was higher (358 ng LDL/mg protein) when the ECs were cocultured with astrocytes than when they were cultured alone (215 ng LDL/mg protein).

The C7-monoclonal antibody, known to interact with the receptor binding domain, totally blocked the binding of LDL to bovine brain capillary ECs. Control irrelevant IgG had no effect on LDL binding (results not shown), suggesting that binding on endothelial cells is mediated by the receptor.

These results indicate that brain capillary ECs at confluence, cultured in a medium containing lipoproteins, express LDL receptors in contrast to what was observed on aortic ECs. Furthermore, there was an increase in the number of the LDL receptors on brain capillary ECs in the presence of astrocytes. Since LDL serves as the principal carrier of exogenous cholesterol to peripheral tissues (Fredrickson et al., 1967), it is possible that the cholesterol content of astrocytes modulates LDL receptor expression on brain capillary ECs.

Cholesterol Content and Binding of LDL on Astrocytes

The ability of the cultured astrocytes to bind and internalize lipoproteins was shown by Pitas et al. (1987). The pretreatment of astrocytes with LPDS led to a time-dependent increase in the binding of [125I]LDL to the cells (Fig. 4), indicating that the cells have modified their metabolism to obtain more exogenous cholesterol. Furthermore, HPLC analysis shows a 32% decrease of the cholesterol content of astrocytes (27.00 ± 3.37 μg cholesterol/mg protein vs 39.25 ± 4.03 μg cholesterol/mg protein, $n = 6$), when they were incubated at 37°C for 36 h in LPDS.

Upregulation of the LDL Receptor

To investigate the role of the astrocytes in the regulation of the binding of LDL to ECs, the experiments described in
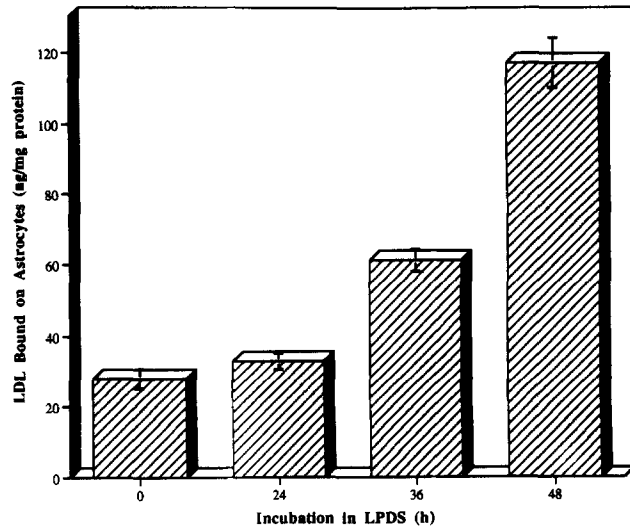

Figure 4. Specific binding of LDL to astrocytes incubated with lipoprotein deficient serum. After maturation (3 wk), astrocytes were incubated at 37°C for indicated times with DME supplemented with 10% LPDS, 2 mM glutamine, and 50 μg/ml gentamycin. At the end of the incubation, [125I]LDL binding was performed at a concentration of 30 μg/ml of [125I]LDL, as described under the experimental procedures. The results are representative of three series of independent experiments.

Fig. 1 were performed. Brain capillary ECs and astrocytes were cocultured for 12 d in a medium containing 15% CS. 36 h before the binding experiments, ECs were transferred onto other astrocytes, and the astrocytes of the coculture were cultured for 36 h in a medium containing 10% LPDS to modify the intracellular metabolism of these cells. After this period, brain capillary ECs and cholesterol-depleted astrocytes in their conditioned medium were again cocultured, and the binding of radiolabeled LDL was performed. As shown in Fig. 5, increased binding of lipoproteins was already manifested 3 h after coculture of the two cell types at 37°C, and the maximum effect (600% of the control binding) was observed at 4 and 5 h. When brain capillary ECs were incubated with cycloheximide during the "induction phase," no increase in LDL receptor expression was observed (data not shown). The toxicity of cycloheximide was assessed by calculating the permeability of sucrose and inulin of the monolayer (Dehouck et al., 1992). No effect of cycloheximide at the concentration of 10 μg/ml was observed.

Preliminary Characterization of the Astrocyte Upregulation LDL Receptor Factor(s) (AUF)

Since these effects in coculture were mediated through a filter that prevented direct cell–cell contact, we hypothesized that soluble factor(s) derived from astrocytes were responsible for inducing the upregulation of the LDL receptor. To test this hypothesis, brain capillary ECs and astrocytes were cocultured for 12 d. 36 h before the binding, brain capillary ECs were transferred onto other astrocytes, and the astrocytes of the original cocultures were fed with a medium containing 10% LPDS. When the astrocyte-conditioned medium was added to the abluminal side of brain capillary ECs, and the ECs were cultured for different times at 37°C, we ob-
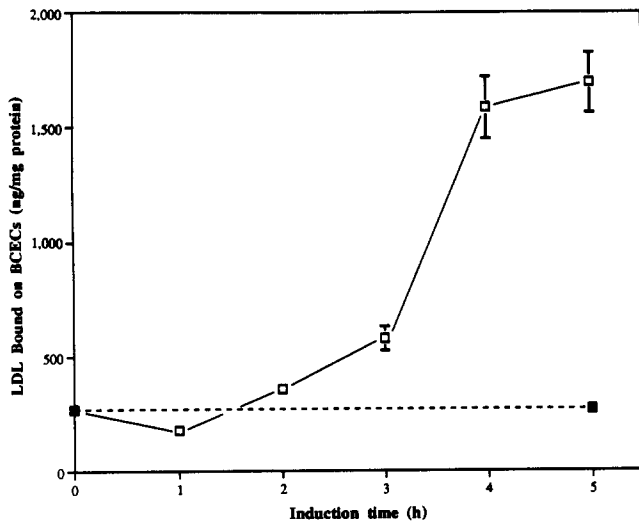
Figure 5. Upregulation of LDL receptor on brain capillary ECs. Specific binding of LDL at 4°C to brain capillary ECs: the upregulation was performed as described in Fig. 1, with astrocytes fed for 36 h with LPDS (□). The control was the binding of LDL to ECs cocultured with astrocytes fed with serum (■), during the "astrocyte LPDS phase." The curves are representative of six series of independent experiments.

served an increase (300%) in the LDL binding at 4°C after 4 or 5 h incubation (Fig. 6).

An estimate of the molecular weight of the AUF(s) was obtained by the following experiments. The astrocytes were cocultured with brain capillary ECs for 12 d. Then they were fed for 36 h with a medium containing 10% LPDS. During
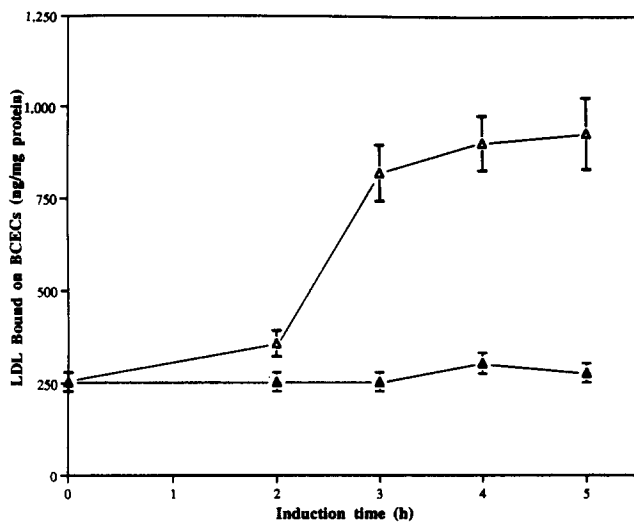

Figure 6. Upregulation of LDL receptors on brain capillary ECs by astrocyte-conditioned medium. Specific binding of LDL at 4°C to brain capillary ECs: the upregulation was performed as described in Fig. 1, but during the induction phase, only the conditioned medium was added to the abluminal face of brain capillary endothelial cells (BCECs). LDL binding was performed to BCECs after incubation at 37°C in the presence of the conditioned medium obtained from astrocytes in LPDS (△) or as a control in FCS (▲). The results are representative of three series of independent experiments.
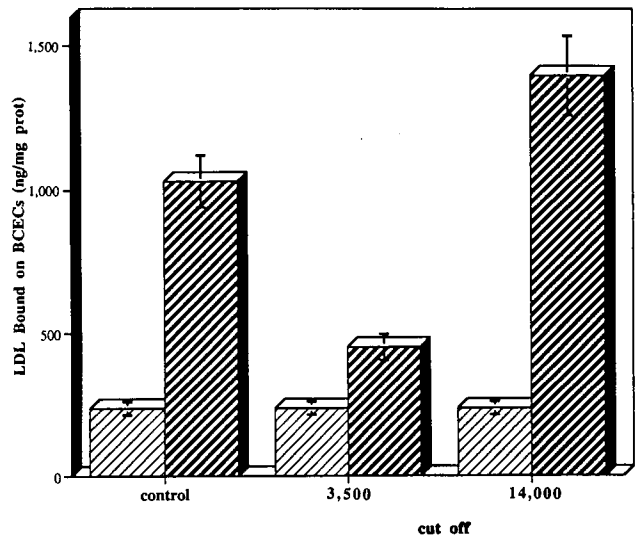

Figure 7. Preliminary characterization of soluble factor(s) secreted by astrocytes. Specific binding of LDL at 4°C to brain capillary ECs: the upregulation was performed as described in Fig. 1, but during the induction phase the two cell types were separated by cut off dialysis bags of 3,500 or 14,000 mol wt. BCECs were incubated during the induction phase with astrocytes fed with LPDS, with or without a dialysis bag (▨), or with astrocytes fed with FCS (▨) as a control. The results are representative of two series of independent experiments.

the induction experiment, dialysis bags with a variable cut-off were used to separate the two cell types. No increase of LDL receptor expression was observed in the coculture with a cut-off membrane of 3,500. In contrast, the upregulation was observed in the coculture with a cut-off membrane of 14,000 (Fig. 7).

Specificity of the Upregulation

To test the cell–cell specificity of these observations, similar experiments were performed with aortic ECs or adrenal cortex ECs, SMC instead of brain capillary ECs, and astrocytes, respectively. Under these conditions, no upregulation on aortic ECs and adrenal cortex ECs was observed when the astrocytes were cultured in LPDS for 36 h (data not shown). Similarly, SMC did not induce an upregulation of LDL receptors on brain capillary ECs (data not shown). Interestingly, when astrocytes cultured alone were fed with LPDS during 36 h and then cocultured with brain capillary ECs that had been cocultured for 12 d with other astrocytes, no upregulation of LDL receptors was observed on brain capillary ECs (Fig. 8). Considered together, these results indicate that the 12 d of the coculture are necessary for enhanced LDL binding. Reciprocal communications between ECs and astrocytes are required to activate the mechanism of the upregulation during the coculture phase.

Furthermore, when binding of acetylated LDL is performed instead of LDL to brain capillary ECs, no upregulation of the scavenger receptor occurs (Fig. 9).

Response of Other Cell Types to AUF(s)

Since the secretion of AUF(s) requires reciprocal communications between brain capillary endothelial cells and astro-
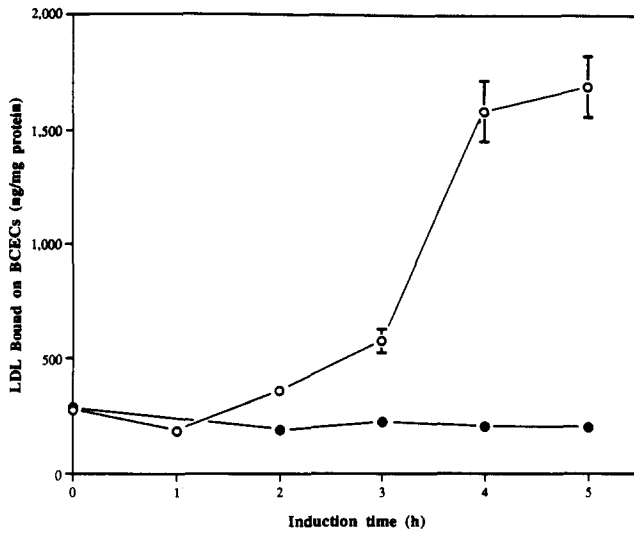
Figure 8. Interaction between brain capillary ECs and astrocytes during the "coculture phase." The upregulation was performed as described in Fig. 1, but astrocytes depleted in cholesterol were not cocultured with BCECs; they were cultured alone. Specific [^{125}I]LDL binding was performed to BCECs incubated with astrocytes either cocultured with BCECs (o) or cultured alone (●).

cytes, the generality of the upregulatory effect of this AUF(s) was tested on other cell types. Astrocytes and brain capillary ECs were grown in coculture for 12 d. Aortic ECs were cultured alone in a medium containing 15% calf serum. Astrocytes, cocultured with brain capillary ECs, were fed for 36 h in a medium containing 15% LPDS. These astrocytes were then cocultured with aortic ECs. No specific binding was observed on aortic ECs cultured alone, but we could see a large increase in the specific binding of the LDL to the aortic ECs when they were in the presence of cholesterol-depleted astrocytes (Fig. 10). The same results were observed when the ex-
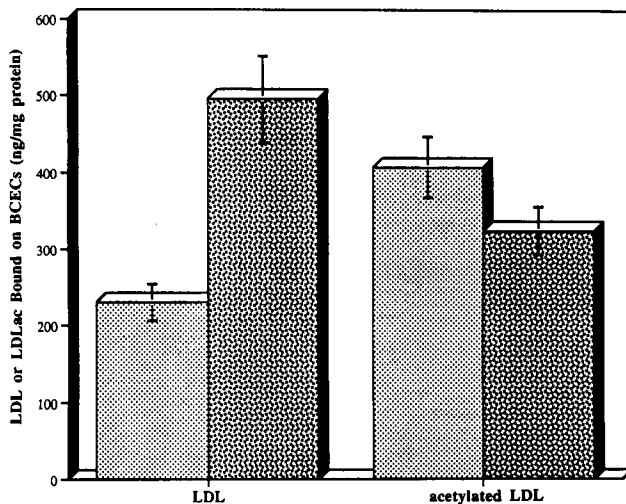

Figure 9. Upregulation of the scavenger receptor on brain capillary ECs. The upregulation was performed as described in Fig. 1. BCECs were incubated for 5 h with astrocytes fed with LPDS (▦) or as a control with FCS (▨). Then specific binding of either LDL or acetylated LDL to brain capillary ECs was performed.
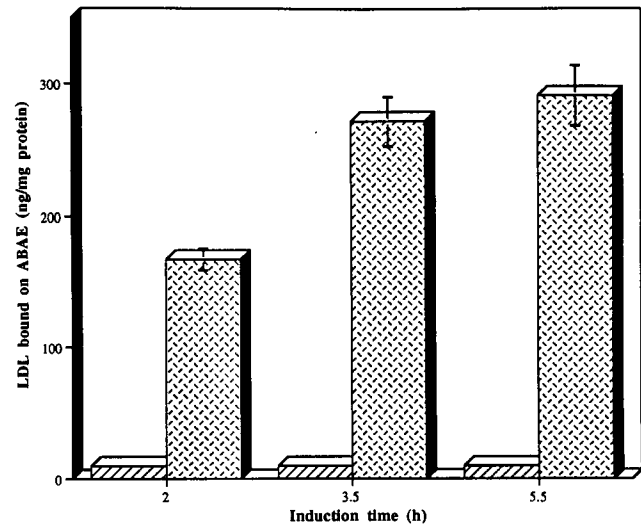
Figure 10. Upregulation of LDL receptor expression on aortic endothelial cell surface. Specific LDL binding to aortic bovine arch endothelial cells (ABAE) after induction of LDL receptor expression: coculture of BCECs and astrocytes and "solo" culture of ABAE were performed. 36 h before the experiment, astrocytes from the coculture were incubated at 37°C with DME supplemented with LPDS or with FCS as a control; then ABAE were incubated for indicated times at 37°C with treated astrocytes and LDL binding was performed to ECs. ABAE were incubated with astrocytes fed for 36 h with LPDS (▨) or with FCS (▨). The results are representative of two series of independent experiments.

periments were performed with SMC instead of aortic ECs (data not shown).

Discussion

The development of this type of coculture system enables the reconstruction of some of the complexities of the cellular environment that exist in vivo while retaining the experimental advantages associated with tissue culture. Thus, the culture medium is shared by both cell populations, allowing humoral interchange without direct cell contact.

LDL Binding to Brain Capillary ECs

In contrast to what was observed on ECs from large vessels, labeled LDL bound specifically to brain capillary ECs that were 5 d postconfluence (as visualized by phase contrast microscopy) and in the presence of a medium containing 15% calf serum. Scatchard analysis of the binding data was used to calculate the K_d for LDL. These data revealed, for the culture with or without astrocytes, the presence on brain capillary ECs of a single class of high affinity LDL binding sites with a K_d of the order of nM. This estimate of affinity is similar to that reported by Vlodasky et al. (1978) in bovine aortic ECs cultured in lipoprotein deficient serum ($K_d = 27$ nM). We have determined (Méresse et al., 1991) by ligand blotting that ECs in culture express an LDL receptor with the same apparent molecular weight as that in vivo (132,000 D). This receptor exhibits the same characteristic properties as the LDL receptor on human fibroblasts (Goldstein and Brown, 1977). Furthermore, we have shown that an antibody

raised against the binding site of the LDL receptor of human fibroblasts (Beisiegel et al., 1981) totally blocks the binding of LDL to bovine brain capillary ECs. These results indicate that we are dealing with an LDL receptor. This LDL receptor was present at the luminal side of ECs. Indeed, labeling of the abluminal receptor could be ruled out, since tight junctions, very well developed in our coculture (Dehouck et al., 1990a), prevent [^{125}I]LDL leak into the abluminal compartment when the experiment is performed at 4°C (data not shown). Since aortic ECs and adrenal cortex ECs cultured in the same medium do not express this LDL receptor at confluence, the culture medium used could not have been responsible for the absence of downregulation observed with brain capillary ECs. This discrepancy may be related to the origin of the ECs (brain capillaries) because several authors have shown that capillary ECs differ from the ECs of other vessels in their biochemical and functional characteristics (Zetter, 1988). The uniqueness of these ECs was previously demonstrated in our laboratory when we showed that angiogenin was mitogenic only for brain capillary ECs (Chamoux et al., 1991).

Our findings also showed that the capacity of ECs to bind LDL is greater when cocultured with astrocytes than in their absence. It is well known that astrocytes are capable of influencing the differentiation of brain ECs in culture, as they are in vivo. Like other cells in the body, astrocytes possess an apo (B, E) receptor, and at the cellular level, this receptor provides a regulated mechanism for supplying cells with cholesterol (Volpe et al., 1978; Pitas et al., 1987). As demonstrated by HPLC analysis and labeled LDL binding, when a complete serum-containing medium was changed to a medium containing 10% LPDS 36 h before the binding experiments, the cholesterol content of the astrocytes was depleted, and the expression of the apolipoprotein (B, E) receptors was upregulated. Under these conditions, we investigated the cholesterol content of astrocytes to see if it modulated the expression of the LDL receptor on the luminal side of brain capillary ECs. In further experiments, astrocytes were incubated for 36 h in LPDS because, after 48 h, cell lysis could be detected in the incubation medium by the determination of the release of lactate dehydrogenase (results not shown).

Upregulation of the LDL Receptor

When astrocytes were pre-incubated in lipoprotein-deficient medium before the induction phase, binding of LDL to endothelial cells was increased a further sixfold. The direct action of LPDS on ECs could be ruled out because LPDS did not increase the LDL binding in 5 h (results not shown). Indeed, the direct action of LPDS on endothelial cells is very well known, and lipoprotein receptor activity of EC is upregulated by exposure to LPDS after a minimum of 24 h. In our experimental conditions, only the albuminal face was in contact with LPDS, and furthermore, upregulation experiments performed with SMC plated instead of astrocytes, or with astrocytes not cocultured with brain capillary ECs, did not enhance LDL receptor expression, even if astrocytes were fed with LPDS during the induction phase. This result indicates that the lipid requirement of astrocytes modulates the expression of endothelial cell LDL receptors. It is well established that coculturing ECs with primary glial cultures

results in marked changes of several endothelial parameters, which suggests that several structural and functional characteristics of cerebral capillaries are determined by the surrounding astrocytes (Joó, 1992). For example, Cancilla and DeBault (1983) have already shown that the proximity in vitro of astrocytes enhances the uptake of neutral amino acids by ECs. These modifications result from specific interactions between brain capillary ECs and brain parenchyma (Risau et al., 1986; Stewart and Wiley, 1981). In our case, we have also shown this kind of specific interaction because substituting astrocytes with smooth muscle cells in coculture did not enhance the expression of the LDL receptors on brain capillary ECs. Moreover, when aortic ECs, originating from peripheral large vessels, or adrenal cortex ECs, originating from peripheral microvessels, were cocultivated with astrocytes, no effect on the upregulation of the LDL receptors was observed when astrocytes were fed with LPDS. Thus, the two close neighbors in vivo, brain endothelium and astrocytes, interact specifically in vitro to induce the expression of the LDL receptor at the luminal side of brain capillary ECs. Furthermore, astrocytes cultured alone do not upregulate LDL receptors on brain capillary ECs during the induction phase. This indicated that a cross-talk takes place between brain capillary ECs and astrocytes during the coculture. The observations that brain ECs can increase the incidence of intramembraneous particle arrays in astrocytes (Tao-Cheng et al., 1990) are in agreement with our results showing that endothelial cells can in turn induce changes in associated glia. The nature of the signal is not known, but Estrada et al. (1990) have shown that a peptide with molecular weight >50,000 derived from cerebral capillary ECs could be involved in the local signaling between cell types that control new vessel formation in development (i.e., brain capillary ECs and astrocytes). These results could explain why with our model, a conditioned medium of astrocytes cultured "alone" did not increase the γ-glutamyl-transpeptidase activity and the electrical resistance of the brain capillary endothelial cell monolayer (Dehouck et al., 1990a).

Furthermore, although brain capillary ECs bind specifically acetylated LDL, astrocytes cocultured with brain capillary ECs for 12 d and fed with LPDS cannot upregulate scavenger receptor expression on brain capillary ECs surface. So, the AUF seems to regulate LDL receptor expression specifically.

AUF(s).

Since these effects were mediated through a filter that prevents direct cell–cell contact, we examined the possible involvement of a soluble factor(s) in this phenomenon. The significant conclusion from the cholesterol-depleted astrocyte conditioned medium experiments is that a soluble factor(s) is involved in the upregulation mechanism. As already stated for the upregulation of the LDL receptor, the molecule(s) could not be a product of the cell lysis that could occur during the incubation with LPDS. The fact that the upregulation is lower when the astrocyte conditioned medium was used could be explained by the secretion of an unstable molecule(s) by astrocytes. The secretion of this factor requires cocultured cells, indicating that the reciprocal differentiation of both cell types might happen. The experiments using dialysis bags allowed only factor(s) with molecular

weights between 3,500 and 14,000 to diffuse to the brain capillary ECs. Since these EC–astrocyte effects are a cross-species (bovine–rat), it is likely to be a fundamental property of the two cell types. AUF liberation is specific and occurs only with cocultured brain capillary endothelial cells and astrocytes.

The experiments using conditioned medium clearly demonstrate that coculture increases the probability of detecting the effects of a short-lived agent produced by astrocytes. That is why we carried out tests to find out if AUF(s), secreted by differentiated astrocytes in the presence of brain capillary ECs, could upregulate the LDL receptor activity in other cell types. The results showed that the AUF(s) secreted by astrocytes triggered the appearance of specific binding on aortic ECs and smooth muscle cells, even when they were cultured at confluence and in the presence of a high concentration of lipoproteins. The ability of the conditioned medium to upregulate receptor levels on other cell types gives us more information about the AUF effect. Indeed, aortic ECs and smooth muscle cells have no intracellular pool of LDL receptor; so an increase in LDL receptor expression requires new synthesis of this receptor. Furthermore, the AUF has no effect if ECs are incubated with cycloheximide. Cycloheximide, known to inhibit peptidyl transferase on eukaryotic large ribosomal subunit, and therefore protein synthesis in the cell, also inhibits the upregulation of LDL receptor expression. These results show that the upregulation is caused by new synthesis of the LDL receptor. Our hypothesis is that the AUF could act on brain capillary endothelial cells and allow the production of a transcriptional factor. The chemical nature of AUF(s) is under investigation.

In conclusion, taken together, these experiments indicate that the occurrence of the LDL receptor on brain capillary endothelial cells at confluence and in the presence of a high concentration of lipoproteins could be explained not only by the originality of the cell type used (brain capillary endothelial cells), but also by the local control of astrocytes. This study confirms the notion that endothelial cells generally not express their final destination-specific differentiated features until these features are induced by local, environment-produced conditions.

Pitas et al. (1987) have proposed a model for cholesterol transport and homeostasis within the central nervous system. Apolipoprotein E, secreted by astrocytes within the brain, transports and redistributes cholesterol via brain interstitial fluid to cells that require cholesterol and express apolipoprotein B,E (LDL) receptors (Hofman et al., 1987). Our results integrated into this model, and they strongly suggest that the cerebral endothelial receptor plays a role in cholesterol transport across the blood–brain barrier. Additional studies are underway to determine if, after binding, lipoproteins are degraded by the endothelial cells or transcytosed in the underlying nervous tissue.

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