Role of membrane-anchored heparin-binding epidermal growth factor-like growth factor and CD9 on macrophages

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Heparin-binding epidermal-growth-factor-like growth factor (HB-EGF) is a potent mitogen for smooth-muscle cells (SMCs) belonging to the EGF family. We have previously determined that HB-EGF is expressed in macrophages and SMCs of human atherosclerotic lesions and that its membrane-anchored precursor, proHB-EGF, also has a juxtacrine mitogenic activity which is markedly enhanced by CD9, a surface marker of lymphohaemopoietic cells. Therefore, when both proHB-EGF and CD9 are expressed on macrophages, they may strongly promote the development of atherosclerosis. In the present study we have investigated the changes in proHB-EGF and CD9 in THP-1 cells during differentiation into macrophages and by the

addition of oxidized low-density lipoproteins (OxLDL) and assessed juxtacrine growth activity of THP-1 macrophages for human aortic SMCs. HB-EGF and CD9 at both the mRNA and the protein level were up-regulated after differentiation into macrophages, and further expression of HB-EGF was induced by the addition of OxLDL or lysophosphatidylcholine. Juxtacrine induction by formalin-fixed growth was suppressed to control levels by an inhibitor of HB-EGF and was partially decreased by anti-CD9 antibodies. These results suggest that co-expression of proHB-EGF and CD9 on macrophages plays an important role in the development of atherosclerosis by a juxtacrine mechanism.

INTRODUCTION

In the development of atherosclerosis, monocyte-derived macrophages take up modified lipoproteins such as oxidized low-density lipoproteins (OxLDL) and become foam cells, which are the major components of the arterial fatty streak [1–3]. It is well known that macrophages release many growth factors and cytokines, and smooth-muscle cells (SMCs) migrate from the media into the intima and promote the development of neointimal lesions [1]. However, the precise mechanism by which macrophages stimulate SMC migration and proliferation is still unclear.

We demonstrated by immunohistochemical analyses that heparin-binding epidermal-growth-factor-like growth factor (HB-EGF) protein is highly expressed in macrophages and SMCs of human atherosclerotic plaque [4]. HB-EGF is a member of the EGF family originally discovered as a fibroblast proliferation factor of macrophage-like cells, and its mitogenic activity for SMCs is similar to that of platelet-derived growth factor (PDGF) [5–8]. The transmembrane precursor, proHB-EGF, reportedly forms a complex with CD9, which was first identified as a surface antigen on lymphohaemopoietic cells and is identical with a 27 kDa diphtheria-toxin-receptor-associated protein [9–12]. Recently we determined that co-expression of proHB-EGF and CD9 on mouse L-cells markedly enhanced the juxtacrine-growth-factor activity of proHB-EGF for EP170.7 cells [13].

In the present study we investigated the expression of proHB-EGF and CD9 during differentiation of THP-1 cells into macrophages and in response to the addition of OxLDL or lysophosphatidylcholine (lysoPC), and assessed juxtacrine growth activity

of macrophages for human aortic SMCs. These observations suggest an important role for macrophages in the progression of atherosclerosis by juxtacrine mechanism.

EXPERIMENTAL

Materials

THP-1 cells were obtained from Japanese Cancer Research Resources Bank (Tokyo, Japan). Human aortic SMCs were purchased from Clonetics (San Diego, CA, U.S.A.). Human HB-EGF cDNA was cloned as described in [5]. Monkey CD9 cDNA was kindly given by Dr. Eisuke Mekada (Institute of Life Science, Kurume University, Fukuoka, Japan) [10]. Human β -actin cDNA was amplified by the following primers:

5'-GACCTGACTGACTACCTCATGAA-3' (591–613)

5'-CTTCATGATGGAGTTGAAGGTAG-3' (871–893)

and subcloned into pBluescript KS. RPMI 1640 and Dulbecco's modified Eagle's medium (DMEM) were purchased from Nacalai Tesque (Kyoto, Japan). Heat-inactivated fetal-calf serum (FCS) was purchased from Gibco (Grand Island, NY, U.S.A.). Egg-yolk lysoPC was purchased from Wako Pure Chemical Co. (Tokyo, Japan). PMA and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [α -³²P]dCTP and [methyl-³H]thymidine were purchased from DuPont–New England Nuclear (Boston, MA, U.S.A.). CRM197, a non-toxic mutant of diphtheria toxin which binds to proHB-EGF and inhibits its activity specifically, was prepared as a specific inhibitor for human HB-EGF as described previously [13]. Anti-(human HB-EGF) antibody, H6, was produced by immunizing rabbits

Abbreviations used: HB-EGF, heparin-binding epidermal-growth-factor-like growth factor; OxLDL, oxidized low-density lipoproteins; lysoPC, lysophosphatidylcholine; SMC(s), smooth-muscle cell(s); PDGF, platelet-derived growth factor; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal-calf serum; PKC, protein kinase C.

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with a synthetic peptide corresponding to amino acids 54–73 of HB-EGF as previously reported [13]. Anti-CD9 monoclonal antibody, ALB-6, was purchased from MBL (Nagoya, Japan).

Cell culture and treatment

THP-1 cells were maintained in RPMI 1640 supplemented with 10% FCS, 100 i.u./ml penicillin and 100 µg/ml streptomycin. PMA was dissolved in ethanol and added to the appropriate medium at a final concentration of 50 ng/ml, and the final concentration of ethanol was kept below 0.1%. The cells were seeded on to 10 cm-diameter dishes at a concentration of $1.0 \times 10^5/\text{cm}^2$ and medium was replaced every 24 h. The cells were washed with PBS and then an indicated amount of lipoproteins or lysoPC was added in serum-free RPMI containing 50 ng/ml PMA.

Human aortic SMCs were seeded at a concentration of $1.0 \times 10^4/\text{cm}^2$ in plastic plates and allowed to attach overnight in DMEM supplemented with 10% FCS. SMCs were used for experiments at passage 4. For experiments, the cells were treated in DMEM containing 2% FCS. Cell viability was determined by Trypan Blue exclusion. The cells were maintained in a humidified atmosphere of air/CO₂ (19:1).

Preparation and modification of lipoproteins

Human LDL ($\rho = 1.019\text{--}1.063 \text{ g/ml}$) was isolated by preparative ultracentrifugation from human plasma of normolipidaemic subjects [14]. After extensive dialysis against 150 mM NaCl/1 mM EDTA, pH 7.4, the protein content of LDL was determined by the method of Lowry et al. [15]. OxLDL was prepared by incubating 400 µg/ml LDL with 5 µM CuSO₄ for 24 h at 37 °C, and stored under nitrogen in the dark at 4 °C. The changes in the electrophoretic mobility of LDL after oxidative modification were evaluated by agarose-gel electrophoresis. The increased electrophoretic mobility of the OxLDL was attributed to the increased negative charge after oxidation (results not shown). The extent of oxidative modification of LDL was evaluated by measurement of thiobarbituric acid-reactive substances [16] (18 nmol/mg of LDL). The phospholipid content of lipoprotein was determined by use of a standard enzymic method [17]. Lipids were extracted from OxLDL, by the method of Folch et al. [18], using chloroform/methanol (2:1, v/v). The amount of lysoPC, which is known to be a major phospholipid component of OxLDL, was analysed by two-dimensional TLC by the method of Rouser et al. [19].

Quantification of mRNA

Total cellular RNA was extracted from THP-1 cells by the guanidinium thiocyanate/phenol/chloroform method [20]. For Northern-blot analyses, total RNA (10 µg/lane) was electrophoresed on 1% agarose, 6.3% formaldehyde gels and transferred to nylon membrane (Hybond N; Amersham Co., Arlington Heights, IL, U.S.A.). For slot-blot analyses, 0.1–10 µg of THP-1 total RNA was denatured and applied directly to membranes. The membranes were UV-cross-linked, prehybridized, hybridized with human HB-EGF, monkey CD9 or human β -actin probes labelled with [α -³²P]dCTP by means of a random-primer-labelling system (Amersham). The blots hybridized with human HB-EGF or human β -actin probes were washed 1 × 20 min in 1 × SSC (0.15 M NaCl/0.015 M sodium citrate) containing 0.1% SDS and 3 × 20 min in 0.1 × SSC containing 0.1% SDS at 65 °C. The blots hybridized with monkey CD9 probes were washed 1 × 20 min in 2 × SSC containing 0.1% SDS and 2 × 20 min in 1 × SSC containing 0.1% SDS at 65 °C.

Hybridized membranes were exposed for 24 h at –80 °C to Kodak XAR-5 film between two intensifying screens. Hybridization signals were quantified by scanning the films with a densitometer and comparing slopes (obtained by linear-regression analysis) of signal areas versus total RNA loaded. We confirmed that each signal of HB-EGF, CD9 and β -actin was detected as a single band by Northern-blot analysis. Since human β -actin mRNA per total RNA remained constant during differentiation of THP-1 by PMA as previously reported [21], HB-EGF and CD9 mRNA levels were expressed as a relative ratio to β -actin.

Detection of proHB-EGF and CD9 on cell surface

THP-1 cells were incubated with 50 ng/ml PMA at 5.0×10^5 cells/6 cm dish for 48 h and then treated with indicated amounts of OxLDL or lysoPC for 24 h. The cells were washed with PBS containing 2 M NaCl to remove any soluble growth factors which might bind to the cell-surface matrix, and twice with PBS [22]. The cells were gently removed from the dish with a rubber policeman, and immediately fixed with 1% paraformaldehyde for 20 min on ice. After washing with PBS, the cells were incubated with anti-HB-EGF antibody, anti-CD9 antibody, or control IgG for 30 min on ice. The cells were then washed twice with ice-cold PBS and incubated with FITC-conjugated goat anti-rabbit IgG or FITC-conjugated goat anti-mouse IgG for 30 min on ice. Then cells were washed twice with ice-cold PBS and analysed using a FACScan flow cytometer (Becton Dickinson and Co., Mountain View, CA, U.S.A.).

Measurement of DNA synthesis in human aortic SMCs

Measurement of DNA synthesis in human aortic SMCs was performed by [*methyl*-³H]thymidine incorporation. For the juxta-crine-growth-factor assay, THP-1 cells were washed with RPMI containing 2 M NaCl to remove soluble growth factors possibly bound to cell-surface heparan sulphate proteoglycans [22], washed twice with RPMI, and fixed for 5 min with 5% buffered formalin to eliminate the effects of newly secreted growth factors, including mature HB-EGF. The formalin-fixed THP-1 cells (5.0×10^3 cells) were washed twice with DMEM containing 2% FCS and then added to SMCs, which had been seeded at 5.0×10^3 cells/well in 96-well plates for 24 h before the experiment. After 36 h, [*methyl*-³H]thymidine was added for a further 5 h at 1 µCi/well. The cells were trypsin-treated and retrieved on to glass-fibre filters using an automatic cell harvester, and [*methyl*-³H]thymidine uptake was assessed in a direct β -radiation counter.

RESULTS

Expression of HB-EGF mRNA and CD9 mRNA during differentiation into macrophages

Changes in HB-EGF and CD9 mRNA levels were studied in THP-1 cells. No detectable HB-EGF mRNA and relatively low CD9 mRNA levels were observed in undifferentiated THP-1 monocytes using Northern-blot analysis (Figure 1, inset). The expressions of HB-EGF and CD9 mRNA were significantly up-regulated during macrophage differentiation by PMA treatment. Human β -actin mRNA per total RNA remained constant during differentiation into macrophages (results not shown). The HB-EGF/ β -actin mRNA ratio quantified by slot-blot analyses significantly increased, peaked at 24 h by 4.5-fold compared with 0-h control values and remained high over 96 h (Figure 1). The CD9/ β -actin mRNA ratio also increased at 24 h (3.7-fold compared with 0 h control values) and remained high over 96 h.

Figure 1 Expression of HB-EGF mRNA and CD9 mRNA during macrophage differentiation

THP-1 cells were exposed to 50 ng/ml PMA, and total RNA was extracted from the cells at the indicated time after the addition of PMA. Time 0 h represents undifferentiated THP-1 monocytes. HB-EGF, CD9 and β -actin mRNA levels were determined by slot-blot analyses as described in the Experimental section. The signal density of HB-EGF or CD9 relative to β -actin was divided by that of the control at each time point. Bars denote S.D. for three experiments. The inset shows a Northern-blot analysis of HB-EGF and CD9 mRNA. A 10 μ g portion of RNA per lane was electrophoresed on 1% agarose/6.3% formaldehyde gel, transferred to a nylon membrane, and hybridized to HB-EGF, CD9 or β -actin probe.

Figure 2 Time course of HB-EGF mRNA induction by OxLDL and lysoPC

THP-1 cells were fed as described in the Experimental section. Differentiated THP-1 macrophages (50 ng/ml PMA for 48 h) were incubated with OxLDL (50 μ g/ml) or lysoPC (5 μ g/ml). Total RNA was extracted at the indicated time and mRNA levels were determined by slot-blot analyses as described in the Experimental section. The corrected signal density for each time point was presented as a relative unit, as described in Figure 1. Bars denote S.D. for three experiments. $\circ$, OxLDL; $\bullet$, lysoPC.

THP-1 macrophages treated with PMA for 48 h expressed both scavenger receptors and CD36, putative receptors for OxLDL, according to flow-cytometric results (not shown). Therefore, OxLDL was added to THP-1 cells between 48 and 96 h after PMA addition.

Figure 3 Dose effects of OxLDL and lysoPC on HB-EGF mRNA

Differentiated THP-1 cells (48 h treatment with PMA) were incubated with the indicated concentrations of OxLDL or lysoPC for 3 h. The doses of OxLDL used were 0–100 μ g/ml, containing 0–10 μ g/ml lysoPC. Slot-blot analyses were performed as described in the Experimental section and Figure 1. Bars denote the S.D. for three experiments. $\circ$, OxLDL; $\bullet$, lysoPC.

Induction of HB-EGF mRNA expression by OxLDL and lysoPC in differentiated THP-1 cells

In order to clarify the changes of HB-EGF expression induced by OxLDL, we examined the dynamics of HB-EGF mRNA levels after OxLDL was added to differentiated THP-1 cells. The HB-EGF/ β -actin mRNA ratio quantified by slot-blot analyses significantly increased after the addition of OxLDL (1.5-fold) at 3 h (Figure 2). Since lysoPC has been reported to be one of the main biologically active components of OxLDL [2], we examined the changes of HB-EGF mRNA expression induced by lysoPC at a concentration equal to that of OxLDL. The HB-EGF/ β -actin mRNA ratio also increased after the addition of lysoPC (1.6-fold) at 3 h (Figure 2). Next, the effects of different doses of OxLDL or lysoPC (concentrations equal to those of OxLDL) on HB-EGF mRNA levels were investigated in differentiated THP-1 cells after 3 h incubation with these agents (Figure 3). The increase in the HB-EGF/ β -actin mRNA ratio was observed in a dose-dependent manner up to 5.0 μ g/ml of lysoPC. The time- and dose-response curves of HB-EGF mRNA levels after the

Figure 4 Time course of CD9 mRNA treated with OxLDL and lysoPC

THP-1 cells were fed as described in the Experimental section. Differentiated THP-1 cells exposed to 50 ng/ml PMA for 48 h were incubated with OxLDL (50 μ g/ml) and lysoPC (5 μ g/ml). Total RNA was extracted at the indicated time, and mRNA levels were determined by slot-blot analyses as described in the Experimental section and Figure 1. Bars denote the S.D. for three experiments. $\circ$, OxLDL; $\bullet$, lysoPC.

Figure 5 Expression of proHB-EGF and CD9 protein on THP-1 cells

THP-1 cells treated with 50 ng/ml PMA for 48 h were incubated with OxLDL (50 μ g/ml) or lysoPC (5 μ g/ml) for 24 h. The cells were washed with PBS containing 2 M NaCl to remove soluble growth factors. After washing with PBS, the cells were incubated with anti-HB-EGF antibody, anti-CD9 antibody or an 'irrelevant' control antibody. Then the cells were incubated with FITC-conjugated second antibodies, and fluorescence intensity was measured by flow cytometry as described in the Experimental section. (A) Expression of proHB-EGF treated with OxLDL (top) and lysoPC (bottom). (B) Expression of CD9 on undifferentiated THP-1 monocytes (top), differentiated THP-1 macrophages (bottom), differentiated THP-1 macrophages treated with OxLDL or lysoPC (bottom). The Figure shows the representative results from three experiments.

addition of OxLDL were nearly the same as those after the addition of lysoPC at the same concentration in OxLDL. In contrast with HB-EGF mRNA levels, CD9 mRNA levels did not change after the addition of OxLDL or lysoPC over 6 h either in response to time (Figure 4) or dose (results not shown).

Expression of proHB-EGF and CD9 protein on THP-1 cells

The expression of proHB-EGF and CD9 protein on the cell surface was analysed by immunofluorescent flow cytometry using anti-HB-EGF antibody, anti-CD9 antibody or control IgG. Figure 5(A) shows the changes in the expression of proHB-EGF on differentiated THP-1 cells treated with OxLDL (top) or lysoPC (bottom). ProHB-EGF was expressed on differentiated THP-1 cells, and a further increase occurred after the addition of OxLDL or lysoPC. The expression of proHB-EGF could not be detected on undifferentiated THP-1 cells (results not shown). In contrast, the expression of CD9 was observed at low, but significant, levels on undifferentiated THP-1 cells (Figure 5B, top) and was markedly increased by macrophage differentiation (Figure 5B, bottom). However, no additional changes were observed after treatment with OxLDL or lysoPC (Figure 5, bottom).

Table 1 Juxtacrine effects of THP-1 macrophages on DNA synthesis in human aortic SMCs

Differentiated THP-1 cells were washed with RPMI containing 2 M NaCl to remove soluble growth factors, fixed with 5% buffered formalin, and then added to SMCs. CRM 197 (10 μ g/ml) or anti-CD9 antibodies (10 μ g/ml) were added to SMCs with fixed THP-1 macrophages. DNA synthesis in SMCs was measured as described in the Experimental section. Data represent means $\pm$ S.D. for three experiments.

Addition	DNA synthesis (fold increase)		
	No antibody	Anti-CD9 antibody	CRM197
None (Control)	1.00 $\pm$ 0.09	0.81 $\pm$ 0.10	0.89 $\pm$ 0.22
THP-1 macrophages	2.20 $\pm$ 0.03	1.68 $\pm$ 0.11	0.92 $\pm$ 0.21

Juxtacrine effects of proHB-EGF and CD9 on differentiated THP-1 macrophages on DNA synthesis in human aortic SMCs

Differentiated THP-1 cells stimulated DNA synthesis in human aortic SMCs by 2.2-fold compared with that of non-treated controls (Table 1), although cells were washed with 2 M NaCl to remove matrix-bound growth factors and fixed with formalin.

Table 2 Juxtacrine effects of THP-1 macrophages treated with lysoPC on DNA synthesis in human aortic SMCs

After 24 h treatment with lysoPC (5 μ g/ml) or none, THP-1 macrophages were washed with RPMI containing 2 M NaCl to remove soluble growth factors, fixed with 5% buffered formalin, and then added to SMCs. DNA synthesis in SMCs was measured as described in the Experimental section. Data represent means $\pm$ S.D. for three experiments.

Treatment	DNA synthesis (fold increase)
None (control)	1.00 $\pm$ 0.07
LysoPC	1.43 $\pm$ 0.08

Furthermore, a specific inhibitor of human HB-EGF, CRM197, completely suppressed DNA synthesis stimulated by THP-1 macrophages to control levels, and anti-CD9 antibodies partially decreased the induced DNA synthesis by 40%. Undifferentiated THP-1 monocytes had no effects on DNA synthesis (results not shown). Next, the effect of fixed THP-1 macrophages treated with lysoPC on DNA synthesis was examined. As expected, lysoPC-treated THP-1 macrophages increased SMC-DNA synthesis by 1.43-fold compared with THP-1 macrophages without lysoPC treatment (Table 2).

DISCUSSION

In the present study we have demonstrated that differentiated THP-1 macrophages express high HB-EGF and CD9 mRNA levels and that OxLDL or lysoPC treatment induces further expression of HB-EGF mRNA. The cell-surface levels of proHB-EGF and CD9 protein on THP-1 cells were also elevated during monocyte-to-macrophage differentiation and were enhanced by both OxLDL and lysoPC. The time- and dose-response curves of HB-EGF mRNA levels after OxLDL treatment were nearly the same as those after lysoPC treatment at the same concentration in OxLDL, suggesting that the effects of OxLDL on HB-EGF may be mainly due to lysoPC. Furthermore, formalin-fixed THP-1 macrophages exhibited juxtacrine growth activity for human aortic SMCs, an effect which was totally inhibited by an HB-EGF inhibitor. A further increase of juxtacrine growth activity was observed in response to the addition of fixed THP-1 macrophages treated with lysoPC. These results suggest that the juxtacrine growth effects of THP-1 macrophages for SMCs are mainly due to proHB-EGF, which is induced by lysoPC.

In early stages of atherosclerosis, monocytes attach to endothelial cells and differentiate to macrophages after infiltrating into arterial subendothelial space [1]. At advanced stages, SMCs migrate from the media into the intima, and macrophages become foam cells by taking up modified lipoproteins such as OxLDL through CD36 and scavenger-receptor pathways [2,23,24]. We previously determined that HB-EGF protein is highly expressed in SMCs and macrophages of human atherosclerotic lesions, which suggests a strong relationship between HB-EGF and the development of atherosclerosis [4]. HB-EGF is a macrophage-derived potent mitogen for SMCs, and its mitogenic activity is similar to that of PDGF [5–8]. We recently reported that proHB-EGF, a membrane-anchored HB-EGF, also has juxtacrine-like biological activity and that CD9 enhances its growth-factor activity by 25-fold using transfectants of mouse L-cells [13]. However, the juxtacrine growth effects of macrophages for SMCs are poorly understood. In the present study THP-1 macrophages stimulated DNA synthesis of human aortic SMCs, although cells were washed to remove matrix-bound HB-

EGF and fixed with formalin to eliminate the effects of newly secreted growth factors, including mature HB-EGF. Unfixed THP-1 macrophages stimulated DNA synthesis by 1.28-fold compared with fixed THP-1 macrophages (results not shown), suggesting that unfixed THP-1 macrophages enhance SMC proliferation by both juxtacrine and paracrine processes. Since we focused on the juxtacrine mechanism, experiments were carried out using fixed THP-1 cells. This juxtacrine growth effect was totally blocked by an inhibitor of HB-EGF and partially by anti-CD9 antibodies. Since it is speculated that CD9 participates in the conformational stability of proHB-EGF on the cell surface [13], anti-CD9 antibodies may reduce the stability of proHB-EGF and decrease juxtacrine growth activity of proHB-EGF. Our results suggest that membrane-anchored HB-EGF and CD9 may participate in the proliferation of SMCs around macrophages and foam cells at localized atherosclerotic lesion.

OxLDL is considered to be generated *in vivo*, and an oxidative modification is catalysed by a phospholipase A₂ activity in the LDL with extensive conversion of phosphatidylcholine into lysoPC, which is a major component of OxLDL [2,3]. Previous studies demonstrated that OxLDL plays an important role in atherogenesis and that its biological activities are mainly due to lysoPC [2,3,25–28]. In the present study, OxLDL and lysoPC increased HB-EGF mRNA of differentiated THP-1 cells in the same time- and concentration-dependent manner, and lysoPC-treated differentiated THP-1 cells increased juxtacrine growth activity compared with differentiated THP-1 cells without lysoPC treatment. Therefore, at an early phase of foam-cell formation, lysoPC may play an important role in the stimulation of HB-EGF expression in macrophages at both mRNA and protein levels, resulting in the enhanced SMC proliferation.

The human monocytic leukaemic cell line THP-1 morphologically changes into macrophages after PMA treatment [29]. During macrophage differentiation, THP-1 cells express scavenger receptors, CD36, apolipoprotein E and lipoprotein lipase, as do macrophages in the arterial wall [21,30,31], and differentiated THP-1 cells accumulate cholesteryl esters when exposed to modified LDL [31,32]. Therefore THP-1 is a useful cell line for studying the properties of monocyte-macrophages in the atherosclerotic process. Since activation of the protein kinase C (PKC) pathway is essential for macrophage-like morphology in THP-1 cells [33,34], increased HB-EGF and CD9 mRNA levels accompanying monocyte-to-macrophage differentiation may be due to direct PKC activation. However, additional effects of OxLDL or lysoPC were observed on steady-state HB-EGF mRNA levels, which suggests that the increase in HB-EGF mRNA induced by these agents is independent of the influence of PMA.

PDGF, another potent mitogenic growth factor for SMCs, is also secreted by macrophages [1,35–37]. PDGF mRNA levels were reportedly increased during monocyte-to-macrophage differentiation, as are HB-EGF levels [36]. However, Ross and co-workers [37,38] reported that PDGF was only present in non-foam-cell macrophages and not in lipid-rich foam cells, as demonstrated by immunohistochemical analysis of human atherosclerotic lesions. These findings suggest that PDGF increases during macrophage differentiation but decreases after foam-cell formation. Our previous immunohistochemical study of HB-EGF in human atherosclerotic plaque indicated that macrophages and SMCs, including foam cells, were strongly positive for HB-EGF protein [4], which is consistent with our present observation *in vitro*. Therefore it is speculated that HB-EGF on macrophages may promote SMC growth more potently than does PDGF, at least in advanced stages of atherosclerosis.

In conclusion, marked stimulation of HB-EGF and CD9 expression was observed in THP-1 cells during macrophage

differentiation at both mRNA and protein levels, whereas THP-1 monocytes expressed no detectable levels of HB-EGF and relatively low levels of CD9. OxLDL enhanced HB-EGF mRNA and membrane-anchored-protein levels on THP-1 macrophages, and this effect is considered to be induced by lysoPC. Since proHB-EGF and CD9 on macrophages play a major role in juxtacrine growth effects for SMCs, further investigation is needed to elucidate the intracellular signals by which they act.

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