Expression and function of ephrin-B1 and its cognate receptor EphB2 in human atherosclerosis: from an aspect of chemotaxis

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ABSTRACT

Although several cytokines and chemokines have been demonstrated to play pivotal roles in the pathophysiological conditions of atherosclerosis, few findings exist regarding the expression and function of cytokine-modulating molecules such as ephrin-Bs and their cognate receptors, EphBs, in human atherosclerosis. Therefore, in the present study, we screened novel genes modulating atherogenesis by cDNA array and quantitatively determined them by real-time RT (reverse transcription)-PCR in human carotid atherosclerotic plaques. Ephrin-B1 and EphB2, key regulators of embryogenesis, were significantly up-regulated in plaques compared with those in adjacent control tissues [ephrin-B1, 0.638 ± 0.106 compared with 0.831 ± 0.152, or 130 % (P < 0.05); EphB2, 1.296 ± 0.281 compared with 2.233 ± 0.506, or 172 % (P < 0.05)]. Immunohistological analysis demonstrated that both ephrin-B1 and EphB2 were expressed in macrophages and T-lymphocytes in plaques as well as in monocytes, T-lymphocytes and arterial endothelial cells isolated from healthy adults. Interestingly, the extracellular domains of ephrin-B1 and EphB2, the expression of which were both enhanced in stimulated THP-1 cells, significantly inhibited spontaneous (22.5 and 27.6 % respectively; P < 0.01) and MCP-1 (monocyte chemoattractant protein-1)-dependent (29.7 and 22.6 % respectively; P < 0.01) migration of monocytes. In conclusion, these results demonstrate that ephrin-B1 and EphB2 are overexpressed in atherosclerotic tissue and might locally regulate cell migration, possibly through modulating cytokine-related chemotactic activity; however, the functional role of these molecules in atherogenesis should be investigated further.

INTRODUCTION

Transmigration of monocytes to the subendothelial space is the initial step in atherosclerotic plaque formation [1]. Their interaction with endothelial cells is mediated by adhesion molecules, such as integrins, and their chemotaxis is directed by chemokines including MCP-1 (monocyte chemoattractant protein-1) [2]. Monocytes are then differentiated into macrophages, the key players in atherogenesis [3]. Under these conditions, numerous inflammatory molecules, including cytokines, are considered to be involved

Key words: angiogenesis, atherosclerosis, cell migration, chemokine, cytokine, embryogenesis.

Abbreviations: AP, alkaline phosphatase; CEA, carotid endarterectomy; DAB, diaminobenzidine; Eph, ephrin receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCAEC, human coronary artery endothelial cell; MCP-1, monocyte chemoattractant protein-1; Pl, atherosclerotic plaque; PO, peroxidase; rN, relatively normal; RT, reverse transcription; SDF-1, stromal-derived factor-1; vWF, von Willebrand factor.

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Ephrins, the key regulators in embryogenesis, have recently gained attention as inflammatory molecules [6]; however, few studies exist describing the expression and function of ephrins in human atherosclerosis. In the present study, we searched for novel molecular mechanisms of human atherogenesis using cDNA arrays including ephrins and their cognate receptors Ephs. We analysed further the expression and function of the final candidate genes in vitro.

Part of this study was presented at the American Heart Associations Annual Scientific Sessions, held in Chicago on 12–15 November 2006, and subsequently published in abstract form [6a].

MATERIALS AND METHODS

Tissue sampling of human carotid arteries
Informed consent was obtained from all of the participants in accordance with the standards established by the Ethical Committee of National Cardiovascular Center, Osaka, Japan. Carotid arteries were obtained from ten male patients (mean age, 58.2 ± 3.4 years), who had experienced recent episodes of stroke and underwent CEA (carotid endarterectomy) [7]. All of the specimens were divided into two parts: one contained an atherosclerotic plaque (Pl) in the intima, and the other lacked an overt atherosclerotic plaque [rN (relatively normal)] in the intima. Post-mortem carotid arterial specimens from patients with non-cardiac death were used as controls for immunohistochemistry.

Cell purification and culture
Mononuclear cells from venous blood of healthy adult volunteers were prepared using Lymphoprep (Axis-Shield PoC). For the immunofluorescence study, monocytes and T-lymphocytes were enriched with counter-flow centrifugal elutriation (RSE elutriation system; Hitachi Kokki) [8]. For RT (reverse transcription)-PCR and cell migration assays, monocytes and T-lymphocytes were enriched with MACS Monocyte Isolation Kit II and Pan T cell Isolation Kit II (Miltenyi Biotec) respectively. Human monocytic THP-1 cells and adult HCAECs (human coronary artery endothelial cells) were purchased from A.T.C.C. and Applied Cell Biology Research Institute respectively.

Antibodies and reagents
The following antibodies were used: mouse anti-CD68 (clone KP1; Dako), mouse anti-CD4 (clone 1F6; Novocastra), mouse anti-CD8 (clone 4B11; Novocastra), rabbit anti-[vWF (von Willebrand factor)] (Dako), rabbit anti-(ephrin-B1) (Santa Cruz Laboratories), goat anti-EphB2 (Sigma), biotinylated anti-(goat IgG) (Dako), Alexa Fluor® 488-conjugated anti-(rabbit IgG) or anti-(goat IgG) (Molecular Probes). For ephrin-B1, a rabbit anti-(ephrin-B1) antibody was used unless otherwise noted. The following recombinant proteins were used: MCP-1 (BioLegend), SDF-1 (stromal-derived factor-1) (PeproTech), ephrin-B1-IgG-Fc and EphB2-IgG-Fc (R&D Systems), and IgG-Fc (Athens Research & Technology).

cDNA macroarray analysis
From the ten pairs of rN and Pl CEA specimens, total RNA was isolated using Isogen reagent (Nippon Gene) [8a]. Four pairs of total RNA were adequate for cDNA membrane arrays: Cytokine/Receptor (634517_HuCytoRec.xls) and Cardiovascular (634503_HuCardio.xls) Atlas Arrays (http://clontech.com/support/tools.asp?product_tool_id=155758&tool_id=155759; Clontech). The membranes were hybridized with the cDNA probe mixture synthesized from total RNA from rN or Pl samples (2 µg). The signals of 552 cDNAs were normalized to that of GAPDH (glyceraldehyde-3-phosphate dehydrogenase), and the candidate genes were chosen according to the following criteria: up-regulation greater than 3-fold in at least one Pl and no down-regulation of less than one-third in any of the four Pl specimens. Potential molecular networks were predicted with the Ingenuity Pathway Analysis program (Ingenuity Systems), and their novelty in relation to atherosclerosis was explored with the PubMed database.

RT-PCR

cDNAs were synthesized from the ten pairs of total RNA, quantitative real-time RT-PCR was performed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems), and the expression levels of each target gene were normalized to those of GAPDH [9–11]. For CXCR2 and CXCR4, the forward primer, reverse primer, and TaqMan probe were designed as follows: CXCR2, 5'-TACATGGCTTGATCAGCAAGGA-3', 5'-GCCCT-GAAGAAGGCAACA-3', and 5'-TGCCCAAAG-ACAGCAGGCTTCTCT-3'; and CXCR4, 5'-GGTGTTGTGGTCCAGTGCC-3', 5'-ATAATGCAA-TAGCAGGACAGGATG-3', and 5'-TCATGGTGG-CCTTATCCTGCGTGGTA-3'.

Assay-on-Demand cocktails were used for ephrin-B1 (Hs00270004_m1; Applied Biosystems), ephrin-B2 (Hs00187950_m1; Applied Biosystems), EphB1 (Hs00174725_m1; Applied Biosystems), EphB2 (Hs00362096_m1; Applied Biosystems), EphB3 (Hs00177903_m1; Applied Biosystems) and EphB4 (Hs00174752_m1; Applied Biosystems). Pre-developed TaqMan assay reagent was used for GAPDH.

Conventional RT-PCR was carried out as follows: preheating for 94°C for 2 min, 35 cycles of amplification, 94°C for 20 s, 60°C for 30 s, and 72°C for 40 s. For
ephrin-B1 and EphB2, the forward and reverse primers were

designed in their separate exons to eliminate genomic DNA amplification: ephrin-B1, 5′-AGCTGCTTGCTAGCAGCACGTT-3′ and 5′-GAGCAGGAA-GATGACGCA-3′; and EphB2, 5′-GACTCCACTCAGGGCACTGC-3′ and 5′-TTGCGTGTAAGACACGCAG-3′. For GAPDH, the following primers were used: 5′-ACCACGATCATGCACTCAGC-3′ and 5′-TCCACACCCGTGTGCAGTA-3′.

Immunohistochemistry

The tissues were fixed with Histochoice (Amresco), paraffin-embedded and cut into in 3 µm sections. To unmask the CD4 or CD8 antigens, sections were autoclaved in 0.01 mol/l citrate buffer (pH 6.0) for 5 min at 121 °C. For detection of anti-(ephrin-B1) (1:300 dilution), anti-vWF (1:200 dilution), anti-CD68 (1:300 dilution), anti-vWF (1:200 dilution), anti-CD4 (1: 40 dilution) and anti-CD8 (1:40 dilution) antibodies, PO (peroxidase)- or AP (alkaline phosphatase)-conjugated Envision systems (Dako) were used. For localization of anti-EphB2 (1:50 dilution), PO-coupled Histofine MAX-(G) system (Nichirei) or AP-coupled LSAB2 system (Dako) with a biotinylated anti-(goat IgG) antibody (1:100 dilution) was employed.

Colour reactions were developed using DAB (diaminobenzidine) (Dako) for PO and New Fuchsin (Dako) for AP. Single immunohistochemistry was always done with DAB. For double immunohistochemistry, incubation of each antibody was serially performed with colour development, first with DAB and then with New Fuchsin. For the immunofluorescence study, monocytes or T-lymphocytes were smeared on to glass slides, and HCAECs were cultured on a Lab-Tek chamber (Nalge Nunc). For HCAECs, a goat anti-(ephrin-B1) antibody (1:10 dilution) was used. The following Alexa Fluor®-conjugated secondary antibodies were used: Alexa Fluor®488 for CD68, CD4, CD8 and vWF, and Alexa Fluor®488 for ephrin-B1 and EphB2. The cells were incubated with a mixture of two primary antibodies and then with a mixture of the corresponding Alexa Fluor®-conjugated antibodies (1:600 dilution). The mounted slides were examined using an Axiophot2 microscope (Carl Zeiss).

Cell migration assay

Transwell chambers (Corning), whose membranes were pre-coated with ephrin-B1–IgG-Fc, EphB2–IgG-Fc or IgG-Fc as a control (5µg/ml, overnight), were inserted into 24-well culture plates containing 600 µl of assay buffer (RPMI 1640 medium with 0.1% heat-inactivated BSA) with or without chemokines (0.1 µg/ml). Cells (1 × 105 cells; 100 µl) were loaded into the chamber and allowed to migrate down into the culture well for 2 h (37 °C; 5% CO2). For monocytes, chamber membranes with a pore size of 8 µm were used. Non-migrating cells were removed by aspiration and scraping with a cotton swab. The migrating cells, which were present in the culture well or attached to the lower surface of the chamber membrane, were simultaneously incubated with Premix WST-1 reagent (Takara) for 3 h (37 °C; 5% CO2).

The photometric A595 of each well was almost linear up to a cell number of 3 × 104: 0.007 of A595 for 3 x 103 cells, 0.028 of A595 for 1 x 104 cells and 0.092 of A595 for 3 x 104 cells. In addition to the examination with human monocytes, THP-1 cells were also examined, using chamber membranes with a pore size of 5 µm. The migrating THP-1 cells were collected from the culture wells and counted using Z2 Coulter counter (Beckman Coulter). Cell migration without chemokines was considered as spontaneous, whereas migration with MCP-1 (for human peripheral monocytes) or SDF-1 (for THP-1 cells) was considered as chemokine-dependent.

Statistical analyses

Results are expressed as means ± S.E.M. with n observations. Statistical analyses were performed using StatView (version 5.0) software (SAS Institute). Differences were considered significant at P < 0.05.

RESULTS

Up-regulation of ephrin-B1 and EphB2 in human carotid atherosclerotic plaques

After analysing the cDNA arrays covering 552 genes, 151 candidate genes were chosen that met with the present criteria (see the Supplementary Table at http://www.clinsci.org/cs/114/cs1140643add.htm). From the candidate genes, we generated 29 molecular networks using the Ingenuity Pathway Analysis program and searched their novelty in relation to atherosclerosis with the PubMed database. Most of these networks included genes related to atherosclerosis, including lipid-metabolism-related genes, such as apolipoprotein E (apoE) and FABP (fatty acid-binding protein), cytokine genes, such as IL-6 (interleukin 6), growth factor genes, including IGF-1 (insulin-like growth factor-1) and HGF (hepatocyte growth factor), and others, such as MMP9 (matrix metalloproteinase 9) and thrombomodulin. Instead of these previously known molecular networks, attention was focussed on one comprising ephrin-Bs and their cognate receptors EphBs, because this biological system has never been reported to be linked with atherosclerosis.

As for the condition of the tissue samples, the ten rN and Pl pairs of cDNAs appeared reliable for gene expression analysis, because the chemokine receptors CXCR2 and CXCR4, molecular markers for atherosclerosis [3,4], were up-regulated in Pl (Figure 1A). Under these conditions, both ephrin-B1 and EphB2 were found to be significantly up-regulated (P < 0.05) in plaques compared with those in adjacent rN (130 and 172 % respectively) (Figures 1B and 1C).
Expression and localization of ephrin-B1 and EphB2 in human samples

In the control arterial specimen, neither ephrin-B1 nor EphB2 was observed, except in the endothelium (Figures 2A and 2B). In the plaque regions, the intima was grossly eroded, as shown by a lack of immunoreactivity for vWF (Figure 2C). Ephrin-B1 was found mainly in macrophages positive for CD68 [12] (Figure 2D), CD4-positive T-lymphocytes (Figure 2E) and CD8-positive T-lymphocytes (Figure 2F). Similarly, EphB2 was detected primarily in CD68-positive macrophages (Figure 2G), CD4-positive T-lymphocytes (Figure 2H) and CD8-positive T-lymphocytes (Figure 2I). Immunofluorescence showed that ephrin-B1 and EphB2 were expressed in monocytes (Figures 3A and 3B), T-lymphocytes (Figures 3C–3F) and endothelial cells (Figure 3G and 3H) isolated from healthy adult humans, which was confirmed by RT-PCR (Figure 4, lanes 1, 3, and 4).

It is interesting to examine whether the activation of monocytes enhanced cellular expression of ephrin-B1 and EphB2. In a preliminary study, where THP-1 cells (1 × 10^5) were activated with 10 nmol/l PMA for 24 h and the expression levels of ephrin-B1 and EphB2 were analysed by real-time RT-PCR, both ephrin-B1 (from 0.93 to 1.35, or by 46%) and EphB2 (from 0.59 to 1.27, or by 119%) were markedly expressed after activation in vitro.

**DISCUSSION**

Ephrins and Ephs are expressed ubiquitously in embryonic tissues and play key roles in morphogenesis [14,15]. In contrast with cytokines and chemokines, ephrins are cell-membrane-bound ligands, and activation of the receptor tyrosine kinases of Ephs regulates cell adhesion and migration. Consisting of 21 members, ephrins and Ephs are highly redundant and their interactions are promiscuous. Among these ephrins and Ephs, ephrin-B1 and EphB2 were found to be up-regulated in human carotid atherosclerotic plaque in the present study. This overexpression of ephrin-B1 and EphB2 in atherosclerotic plaques can be explained by massive infiltration of macrophages expressing ephrin-B1 and EphB2. However, it is also possible that the expression of ephrin-B1 and/or EphB2 might be up-regulated in a single macrophage, because an additional experiment demonstrated that expression of these molecules could be enhanced in THP-1 cells after cell stimulation in vitro. Armstrong et al. [16] reported the overexpression of the ephrin-A5 (EFNA5) gene in human abdominal aortic aneurysm. Other ephrins and Ephs, including ephrin-A5, might also be expressed in macrophages and T-lymphocytes in human atherosclerotic plaques in carotid arteries; however, the present study is the first to demonstrate the up-regulation of ephrin-B1 and EphB2 in human atherosclerotic tissue.

In the present study, ephrin-B1 and EphB2 were also expressed in the monocytes, T-lymphocytes and endothelial cells from healthy adult humans. Although Yu et al. [17] reported ephrin-B1 expression in mouse spleen monocytes, their functional significance on monocytes and other-related cells has not been clarified well. In the present study, we found that the extracellular domains of ephrin-B1 and EphB2 both inhibited spontaneous and...
chemokine-dependent migration of monocytes as well as THP-1 cells. Similar inhibitory effects of ephrin-B1 and EphB2 on SDF-1-induced chemotaxis are known for other cell types, including ephrin-B1 in Jurkat T-lymphocytes [18] and EphB2 in cerebellar granular cells of mouse embryo [19]. This inhibitory effect of EphB2 is considered so-called reverse signalling [14]. Therefore ephrin-B1 and EphB2 are regarded as new functional cell-surface molecules on monocytes/macrophages [20] even in the process of atherosclerosis.

Through cell-to-cell interactions with neighbouring cells, such as endothelial cells and T-lymphocytes, ephrin-B1 and EphB2 are considered to locally modulate chemotaxis of monocytes/macrophages conditioned by soluble factors such as chemokines. Ephrin-B1 and/or EphB2 on normal endothelial cells might inhibit monocyte transmigration and, therefore, the loss of these molecules due to intimal erosion may facilitate monocyte recruitment in atherosclerotic plaques (Figure 2C).

We have demonstrated in the present study that the expression of ephrin-B1 and EphB2, which might modulate MCP-1-dependent chemotaxis, was augmented in both macrophages and T-lymphocytes in plaques. This suggests that the expressed ephrin-B1 and EphB2 might modulate MCP-1-induced cell transmigration. From a therapeutic point of view, it is interesting

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**Figure 2** Expression of ephrin-B1 and EphB2 in control and atherosclerotic carotid arteries by immunohistochemistry

(A–C) Single immunohistochemistry. (A, B) Control arteries. Note the slight signal in endothelium (arrows). (C) Atherosclerotic arteries. Note that the intima, indicated by an arrow, was scarcely preserved, and inflammatory cells infiltrated, as indicated by arrowheads. (D–I) Double immunohistochemistry for atherosclerosis. DAB (brown)/New Fuchsin (red). The insets show the higher-power field indicated by the arrows. NC, necrotic core. The arrowhead in (G) indicates the lamina elastica interna. Cell nuclei were stained with haematoxylin. Scale bar, 20 \( \mu \text{m} \).
to consider whether ephrin-B1 and EphB2 could be target molecules for alleviating the development of atherosclerosis. HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase inhibitors might enhance activity of these molecules because both ephrin-B1 and Eph-B2 could be partially regulated by the Rho-kinase system which can be altered by HMG-CoA reductase inhibitors [14,15]. However, the complete functional role of ephrin-B1 and EphB2 in atherogenesis is not yet clear. Indeed, it has been shown that ephrin-B1 and Eph-B2 are involved in angiogenesis, which may enhance atherosclerosis [15], and that they induce T-cell costimulation, triggering adaptive immunity [6]. It might even be that vascular expression of ephrins mediates enhanced retention of inflammatory cells in the lesions, thereby stimulating subendothelial inflammation.

There are several limitations of the present study. First, we compared the expression of mRNA in plaque tissue and adjacent relatively normal tissue, which might contain early atherosclerotic components. It is true that completely normal tissue should be used as control; however, even under these conditions, ephrin-B1 and Eph-B2 should be significantly up-regulated in plaque tissue. Secondly, the in vivo functions of ephrin-B1 and Eph-B2 are still unclear, although both exhibited inhibitory activities against MCP-1-dependent migration in vitro. Further studies will be required to demonstrate other functional activities of ephrin-B1 and Eph-B2 in the course of human atherosclerosis. Thirdly, with immunohistochemistry, we did not show negative controls for antibodies against ephrin-B1 and EphB2 because several human cells, including Jurkat cells and CGM1 cells, possess ephrin-B1 and EphB2 by RT-PCR. To overcome this limitation, we performed not only immunohistochemistry, but also RT-PCR in which the forward and reverse primers for both ephrin-B1 and EphB2 were designed in the separate exons, thus eliminating non-specific amplification from genomic DNA.

In conclusion, the present study demonstrates the significant expression of ephrin-B1 and EphB2 in macrophages and T-lymphocytes in atherosclerotic lesions as
Inhibitory effects of ephrin-B1 and EphB2 on the transmigration of human monocytc cells

(A) Spontaneous migration of human monocytes. (B) MCP-1-dependent migration of human monocytes. (C) Spontaneous migration of THP-1 cells. (D) SDF-1-dependent migration of THP-1 cells. The levels of migration with IgG-Fc were 8.0% (A), 7.0% (B), 1.1% (C), and 11.1% (D) of inputs (1×10^5 cells). IgG-Fc, control; ephrin-B1, ephrin-B1-IgG-Fc; EphB2, EphB2-IgG-Fc. Results are means ± S.E.M. (n = 6), and were analysed by one-factor ANOVA and Dunnett's test. *P < 0.01 compared with IgG-Fc (control).

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