Localization of CD9, an Enhancer Protein for Proheparin-Binding Epidermal Growth Factor–Like Growth Factor, in Human Atherosclerotic Plaques

Possible Involvement of Juxtacrine Growth Mechanism on Smooth Muscle Cell Proliferation


Abstract—Heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF), a member of the EGF family, has a potent mitogenic activity for vascular smooth muscle cells (SMCs). We previously reported that HB-EGF is involved in atherogenesis of human aorta and coronary arteries. ProHB-EGF (the membrane-anchored form of HB-EGF) has also been demonstrated to possess a mitogenic activity, which is ≈30-fold increased when coexpressed with CD9 in mouse L cells. Thus, in the process of atherogenesis, CD9 may be involved in the proliferation of SMCs. We immunohistochemically investigated the localization of CD9 and proHB-EGF in the human aorta and coronary arteries. In normal aorta and coronary arteries, CD9 immunostaining was virtually negative, whereas proHB-EGF immunostaining was positive, especially in the arteries of babies. In contrast, in atherosclerotic lesions, some intimal SMCs were strongly positive for CD9 and proHB-EGF immunostaining. The juxtacrine growth activities of human aortic SMCs were inhibited in vitro by adding neutralization antibodies for CD9 or adding the specific inhibitor of HB-EGF. Besides, coexpressed CD9 and proHB-EGF cells markedly incorporated [3H]thymidine into the SMCs. CD9 is localized immunohistochemically in the SMCs of the atherosclerotic aorta and coronary arteries. CD9, when coexpressed with proHB-EGF, enhances proHB-EGF activities for SMC growth in a so-called juxtacrine manner in vitro and may be involved in atherogenesis. (Arterioscler Thromb Vasc Biol. 2000;20:1236-1243.)

Key Words: vascular smooth muscle cells • CD9 • heparin-binding epidermal growth factor–like growth factor • atherosclerosis

The proliferation of vascular smooth muscle cells (SMCs) in the intima of arteries is one of the most important events in plaque formation1 and in coronary restenosis after balloon angioplasty.2 Heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF), a member of the EGF family, has been identified as a potent mitogen for SMCs that is comparable to platelet-derived growth factor.3 Localization and function of HB-EGF have been demonstrated in human hepatocellular carcinoma,4 gastric mucosa,5 wound fluid,6 and balloon-injured rat carotid arteries.7 Moreover, we have reported that HB-EGF is produced by macrophages and SMCs in the human aorta and coronary arteries.9 The HB-EGF precursor (proHB-EGF) is expressed on the cell membrane as a membrane-anchored form and can be cleaved to yield a mature biologically active form (soluble HB-EGF).10 HB-EGF released by SMCs showed mitogenic function in an autocrine and a paracrine fashion.11 Furthermore, proHB-EGF has also been demonstrated to have a mitogenic activity under the condition of cell-to-cell contact12 by a so-called juxtacrine mechanism.13 The juxtacrine growth factor activity of proHB-EGF in mouse L cells is dramatically upregulated ≈30-fold when coexpressed with CD9,12 which was first identified as a cell surface antigen on lymphohematopoietic cells.14,15 Thus, in the process of atherogenesis, CD9 may be involved in the proliferation of SMCs. In the present study, we investigated the immunohistochemical localization of CD9 and proHB-EGF in human coronary arteries and aorta and examined in vitro the effects of CD9 on the proliferation of human aortic SMCs.

Methods

Antibodies Against CD9 and HB-EGF

Antibodies recognizing mature and proHB-EGF were produced by immunizing rabbits with synthetic peptide H-1 (HB-EGF precursor C-terminal residue, proHB-EGF–specific cytoplasmic domain) and...
H-6 (extracellular domain). The H-1 antibody does not cross-react with mature HB-EGF by Western blotting but immunoprecipitates 35S-labeled proHB-EGF. An antibody recognizing CD9 that can be used in a paraffin section was kindly provided by Dr Mekada, Kurume University, Kurume, Japan. This monoclonal antibody was isolated by immunizing BALB/c mice with Vero cell membrane. Immunoprecipitation and immunoblotting studies revealed that this antibody binds to a membrane protein of 27 kDa, identical to CD9.

Immunohistochemical Staining on Paraffin Sections
Thoracic descending aorta and coronary arteries were obtained from 34 autopsied (18 atherosclerotic and 16 nonatherosclerotic) individuals within 5 hours after death with the informed consent of the bereaved families. Tissues were fixed with 10% buffered formalin for 4 to 6 hours at 4°C. After they were washed in 0.01 mol/L PBS, the tissues were decalcified with 0.5 mol/L EDTA solution (Wako) for 3 days at 4°C and embedded in paraffin.

HB-EGF immunohistochemical staining were performed as described in our previous study. A positive reaction was visualized with 3-amino-9-ethylcarbazol. For CD9 immunohistochemical staining, sections were treated with 10% normal goat serum. A 3-step immuno–alkaline phosphatase method was performed with use of a mouse anti-CD9 antibody (0.7 µg/mL), biotinylated goat anti-mouse immunoglobulins, and alkaline phosphatase–labeled streptavidin (DAKO). A positive reaction was visualized with new fuchsin chromogen. For the identification of SMCs and proliferating cells, a mouse monoclonal antibody against an α-isoform of SMC actin (Nichirei) and a mouse monoclonal antibody against a proliferating cell nuclear antigen (PCNA, PC10, DAKO) were applied by using the avidin-biotin complex method (ABC, Vector). A positive reaction was visualized in peroxidase substrate solution containing diaminobenzidine (Zymed). For double immunostaining of CD9 and SMCs or macrophages, an indirect immunofluorescence method was performed with an anti-CD9 antibody (0.4 µg/mL) and a rhodamine-conjugated anti-mouse IgG antibody (5 µg/mL, Cappel) and with an anti-SMC antibody or an anti-macrophage antibody (HAM56, DAKO) and an FITC-conjugated anti-mouse IgG antibody (5 µg/mL, Cappel).

Morphometric analyses were performed on the tissues from the 18 autopsied individuals with macroscopic plaques. To elucidate the role of CD9 in SMCs, we investigated the regions with diffuse intimal thickening (DIT) from 3 different portions of each aorta, because it is too complicated to exclude the effects of macrophages in the plaque. The intima of the DIT regions was divided into quarters from the surface to the internal elastic lamina (IEL). Immunostaining of CD9 or proHB-EGF was evaluated in the bottom quarter (the part near the IEL) and in the other 3 quarters (the parts far from the IEL). For 1 individual, we observed at least 1000 cells. The ratio of immunohistochemically positive cells was shown as an average for the 18 individuals. Statistical analyses were performed by paired Student t test.

Double Immunofluorescent Staining on Cultured SMCs
Human aortic SMCs were obtained from KURABOU. These SMCs were fixed with 4% paraformaldehyde for 10 minutes on ice. The
SMCs were applied with 5% swine serum, followed by an anti–HB-EGF antibody (H-1) (4 \( \mu \)g/mL), a wash in PBS, and a rhodamine-conjugated swine anti-rabbit immunoglobulin antibody (DAKO). After another wash in PBS, 5% rabbit serum, an anti-CD9 antibody (0.4 \( \mu \)g/mL), and an FITC-conjugated rabbit anti-mouse IgG antibody (Cappel) were used. These sections were observed by use of a laser scanning confocal microscope (LSM-GB200, Olympus).

**Juxtacrine Growth Factor Activities of Cultured SMCs**

The juxtacrine growth factor activity of SMCs was measured as follows. SMCs at confluence were washed with 2 mol/L NaCl in PBS to remove matrix-bound growth factors and fixed with 5% buffered formalin to eliminate the effects of newly secreted growth factors, including mature HB-EGF. The formalin-fixed SMCs in DMEM/1% FCS were supplemented with an anti-CD9 antibody (10 \( \mu \)g/mL), CRM 197 (2 \( \mu \)g/mL), or a mouse IgG (10 \( \mu \)g/mL). Then, the fixed cells were added at a concentration of 5\( \times \)10^3 cells per well on the SMCs, which had been seeded at 5\( \times \)10^3 cells per well in 96-well plates for 24 hours before the experiment. After 18 hours, [\( ^{3} \)H]thymidine was added at 1 \( \mu \)Ci per well for a further 6-hour incubation. [\( ^{3} \)H]Thymidine uptake was assessed in a direct \( \beta \)-radiation counter. CRM 197 is a nontoxic mutant of diphtheria toxin, which inhibits HB-EGF and proHB-EGF specifically but not other EGF receptor ligands. An anti-CD9 monoclonal antibody, ALB6 (MBL) was applied to neutralize CD9. The neutralization activity of this antibody on the juxtacrine growth activity of proHB-EGF was previously confirmed.

**Juxtacrine Growth Stimulation on Cultured SMCs**

Mouse L, LC, LH, and LCH cells, described previously, were plated at 1\( \times \)10^5 cells per well in 24-well plates and incubated for 24 hours before washing and fixation. LC cells, LH cells, and LCH cells were confirmed to express CD9, HB-EGF, and both CD9 and HB-EGF, respectively. The cells were washed with 2 mol/L NaCl and fixed with 5% buffered formalin for 5 minutes. The fixed cells were washed twice with DMEM/2% FCS. Then, SMCs at 5\( \times \)10^4 cells per well were added in coculture. After 18 hours, [\( ^{3} \)H]thymidine (1 \( \mu \)Ci per well) was added to the wells, and the cocultured cells were incubated for 6 hours. SMCs were harvested and analyzed for incorporation of [\( ^{3} \)H]thymidine into DNA.

**Results**

**Immunohistochemical Analysis of ProHB-EGF and CD9 in Nonatherosclerotic Arterial Walls**

In the aorta of a 6-month-old baby, CD9 immunoreactivity in the intima and in the media was negative (Figure 1a). In contrast, an intense immunostaining of proHB-EGF (H-1, Figure 1b) was detected in medial SMCs. H-6, which recognizes proHB-EGF and mature HB-EGF, demonstrated the same staining result (data not shown). In the coronary arteries of babies, immunostaining of CD9 was also negative, whereas that of proHB-EGF was positive.

In the aorta (Figure 1c) and coronary arteries (Figure 1d) of a 54-year-old man, the thickness of the intima was physiologically increased. CD9 immunoreactivity was virtually negative, whereas proHB-EGF immunoreactivity was positive in the media and in the intima (not shown). In the aorta and coronary arteries from the 5 young autopsied individuals (aged 0.5 to 15 years), CD9 immunoreactivity was virtually...
negative. CD9 immunostaining was also negative or weakly positive in the aorta and coronary arteries of the other 11 nonatherosclerotic individuals (aged 18 to 63 years). Taken together, CD9 did not demonstrate serial changes in medial or intimal immunoreactivity according to aging or physiological DIT, which have been demonstrated with HB-EGF.

Immunohistochemical Analysis of ProHB-EGF and CD9 in Atherosclerotic Arterial Walls

In the aortas of the atherosclerotic individuals (n=18), immunostaining of CD9 was strongly positive in the plaque and weakly positive in the media (Figure 2a). CD9-positive cells were mainly localized on the plaque shoulder and the fibrous cap and around the IEL. In the coronary arteries (Figure 2b), the plaque lesions showed an intense immunoreactivity to CD9. CD9 immunoreactivity was positive in almost all hypercellular plaques. In addition to the plaque, CD9-positive regions were sometimes detected near the IEL in the DIT region of another section from the same individual (Figure 2c).

For identification of cell types in the plaques, immunostaining of CD9 and SMCs was performed. A set of mirror-image sections (Figure 3a and 3b) demonstrated that the CD9-positive cells around the IEL and in the cap lesion were mainly SMCs. Double immunostaining of CD9 and SMCs revealed that some but not all of the SMCs (Figure 3c and 3d) possessed CD9 immunoreactivity. Most macrophages also had CD9 immunoreactivities (Figure 3e and 3f) in macrophage-rich plaques.

In the arterial wall with plaques, immunoreactivities to proHB-EGF were detected in the media, in the plaque, and near the IEL, whereas immunoreactivities for CD9 seemed to be more predominantly localized just around the IEL (Figure 4a and 4b) than were those for proHB-EGF. To confirm this phenomenon, we investigated the DIT regions of the atherosclerotic individuals, because it is too complicated to exclude the effects of macrophages in the plaque. In the DIT regions of the individuals with aortic atherosclerosis (Figure 4c and 4d), SMCs had CD9 and proHB-EGF immunoreactivities just near the IEL, whereas those localized away from the IEL to the luminal side had no CD9 immunoreactivity (Figure 4c). In contrast, immunostaining of proHB-EGF was still positive in these SMCs localized away from the IEL (Figure 4d). Morphometric analysis showed that CD9-positive SMCs were more predominantly localized near the IEL compared with the localization of proHB-EGF immunostaining (Table). Near the IEL, some cells adjacent to CD9-positive cells had...
PCNA immunoreactivities (Figure 4e). CD9 may be involved in the proliferation of SMCs in a juxtacrine manner in vivo.

Juxtacrine Growth Factor Activities of Cultured SMCs

Human aortic SMCs in culture were stained positively with anti-CD9 and anti–proHB-EGF antibodies (Figure 5a and 5b). On the surface of SMCs, CD9 and proHB-EGF seemed to be coexpressed consistently. We examined the inhibitory effects of an anti-CD9 antibody on the juxtacrine growth of SMCs. Juxtacrine growth stimulation of SMCs was performed by adding formalin-fixed SMCs. The open bars of Figure 5 show that anti-CD9 antibody and CRM 197 had little effect on the autocrine and paracrine growth activity of SMCs in the absence of added fixed cells. By adding fixed cells, SMC thymidine incorporation was markedly increased (closed bar). These juxtacrine growth stimulations were inhibited by an anti-CD9 antibody to an extent similar to that seen with CRM 197. This experiment showed that some molecules on the surface of SMCs stimulated SMC growth by the juxtacrine pathway and that CD9 and HB-EGF were involved in this pathway. To examine the effect of CD9, proHB-EGF, and other cell surface molecules on the proliferation of SMCs, thymidine uptake of SMCs was analyzed by using cells transfected with CD9 and HB-EGF and null cells.
Percentage of CD9- or ProHB-EGF–Positive Cells in Intima of DIT Lesion of Aorta

<table>
<thead>
<tr>
<th></th>
<th>Near IEL</th>
<th>Far From IEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD9-positive cells</td>
<td>43.7±20.2</td>
<td>5.0±5.7*</td>
</tr>
<tr>
<td>ProHB-EGF–positive cells</td>
<td>58.8±18.4</td>
<td>45.2±18.7†</td>
</tr>
</tbody>
</table>

Values are mean±SD. *P<0.0001 and †P<0.005 vs corresponding value near IEL.

(Figure 6). CD9-expressed cells did not show the juxtacrine growth activity, but coexpression of CD9 and proHB-EGF markedly stimulated the juxtacrine growth activity of SMCs. Moreover, compared with L cells, proHB-EGF–expressed cells had little juxtacrine growth activity. Because L cells do not express CD9, this phenomenon shows that the surface molecules on L cells do not enhance the juxtacrine activity of proHB-EGF compared with CD9.

Discussion

CD9, which was first identified as a cell surface antigen on lymphohematopoietic cells,14,15 is a major cell surface protein in pre-β cells, platelets, and activated T lymphocytes.21,22 CD9 is also expressed on the cell membrane of monocytes and nonhematopoietic tissues, including SMCs, fibroblasts, various cancers, and the nervous system.15,23 In the present study, we have demonstrated that CD9 was expressed in the human aorta and coronary arteries, particularly in those with atherosclerotic lesions, and that some of the SMCs possessed a CD9 immunoreactivity. Recently, Scherberich et al24 showed that cultured SMCs express CD9 and that the levels of CD9 in the proliferative phenotype are ~2-fold the levels in the contractile phenotype.

CD9 belongs to the tetraspan superfamily of molecules that have 4 transmembrane domains. CD9, like other tetraspans, was implicated in the adhesive and aggregatory properties of platelets21,22 and other hematopoietic cells, such as the attachment of neutrophils to endothelial cells.25 Other functions besides the adhesion of cells have been implicated. CD9 inhibited the motility of several kinds of tumor cells.26 The CD9 molecule is associated with the β1 chain of very late–acting integrins and regulates integrin signaling.27–29 CD9 promoted the proliferation of Schwann cells.30 Overexpression of CD9 in CHO cells induced the proliferation of these cells.31 Moreover, a tight interaction of CD9 and proHB-EGF was implied by the ability of anti-CD9 antibodies to coprecipitate these 2 molecules.32 Higashiyama et al12 previously demonstrated that proHB-EGF–transfected mouse L cells showed a growth factor activity under the condition of cell-to-cell contact. In contrast, SMCs cultured in contact with LCH cells expressing CD9 and proHB-EGF markedly induced thymidine incorporation. The results are shown as mean±SD. *P<0.001 vs LH cell (n=4). Statistical analyses were performed by paired Student t test.
In the present study, we demonstrated in vitro that SMCs promoted SMC growth in a juxtacrine manner, that CD9 and proHB-EGF were involved in the juxtacrine growth mechanism, and that coexpression of CD9 and proHB-EGF markedly stimulated SMC juxtacrine growth. Immunohistochemical analyses showed that some CD9-positive SMCs were localized adjacent to PCNA-positive SMCs. CD9 may promote juxtacrine growth activities in vivo. In atherogenesis, CD9 might have functions other than the proliferation of SMCs. Further investigation may reveal the roles of CD9 in the migration of SMCs and in the apoptosis of the intimal cells, because CD9 is involved in motility and cell survival.34 On the other hand, activated macrophages secrete many kinds of mitogens and induce the proliferation and migration of SMCs in atherosclerotic lesions. We have also shown that juxtacrine growth activities of macrophages for SMCs were upregulated during differentiation into macrophages and that coexpression of CD9 and HB-EGF on macrophages plays a role in atherogenesis.35

Because the juxtacrine growth mechanism operates only under the condition of cell-to-cell contact, it has an advantage over the paracrine or the autocrine growth mechanism regarding the specificity in the activation of targeted cells. In the present study, we demonstrated that the juxtacrine mechanism may be involved in the proliferation of SMCs. ProHB-EGF is one of the membrane-anchored proteins and has a juxtacrine growth activity. Transforming growth factor-α, tumor necrosis factor-α, colony stimulating factor-1, and the stem cell factor/c-kit ligand also possess juxtacrine activities.36,37 These molecules should be evaluated from the viewpoint of a juxtacrine growth activity. In the present study, juxtacrine pathways other than proHB-EGF were predicted in the SMCs, because the juxtacrine growth activities of SMCs were not completely inhibited by CRM 197.

In conclusion, CD9 is involved in the process of atherogenesis. One of the mechanisms of CD9 in atherogenesis is the mechanism by which CD9 promotes the proliferation of SMCs as an enhancer of proHB-EGF, probably by the juxtacrine growth pathway. The activity enhancer of membrane-anchored growth factor is a new concept, which might become a key point in explaining the mechanism of atherogenesis.

Acknowledgments

This study was supported in part by a grant-in-aid to Y.M. (No. 04040085) from the Japanese Ministry of Education, Science, Sports, and Culture and a grant-in-aid for Cancer Research to S.H. and N.T. (No. 07273108). We wish to acknowledge Toshifumi Oh-ito at Fukuoka University, Fukuoka, Japan, for providing anti-CD9 antibodies. We wish to acknowledge Toshifumi Oh-ito for providing anti-CD9 antibodies.

References

21. Jennings LK, Fox CF, Kouns WC, McKay CP, Ballou LR, Schultz HE. The activation of human platelets mediated by anti-human plate-


Localization of CD9, an Enhancer Protein for Proheparin-Binding Epidermal Growth Factor–Like Growth Factor, in Human Atherosclerotic Plaques: Possible Involvement of Juxtacrine Growth Mechanism on Smooth Muscle Cell Proliferation


Arterioscler Thromb Vasc Biol. 2000;20:1236-1243
doi: 10.1161/01.ATV.20.5.1236

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/20/5/1236

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org/subscriptions/