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Cleavage of Apolipoprotein E by Membrane-Type Matrix Metalloproteinase-1 Abrogates Suppression of Cell Proliferation

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Apolipoprotein E (apoE) in a human fetal brain cDNA library was identified, using the expression cloning method, as a gene product that formed a complex with latent matrix metalloproteinase (MMP)-2. Co-expression of membrane-type MMP-1 (MT1-MMP) with apoE in HEK293T cells reduced the amount of apoE secreted into the culture medium, whereas cell-associated apoE core protein was not affected. Incubation of native apoE protein with recombinant MT1-MMP resulted in the cleavage of apoE. Recombinant apoE protein fused to glutathione S-transferase (apoE-GST) was cleaved by MT1-MMP at the following peptide bonds; T^{85}-M^{86}, K^{93}-S^{94}, R^{246}-L^{247}, A^{255}-E^{256} and G^{296}-L^{297}. HT1080 cells transfected with the apoE gene, which express endogenous MT1-MMP, secreted a low level of apoE protein and its cleaved fragments, and treatment with MMP inhibitor BB94 induced accumulation of apoE and retardation of cell proliferation. Addition of apoE-GST protein to the culture of HEK293T cells suppressed cell proliferation, and stable transfection of the MT1-MMP gene partly abrogated the suppression. These results suggest that cleavage of apoE protein by MT1-MMP abrogates apoE-mediated suppression of cell proliferation.

Key words: apolipoprotein E, cell proliferation, cleavage, MMP, MT1-MMP.

Abbreviations: apoE, apolipoprotein E; GST, glutathione S-transferase; MMP, matrix metalloproteinase; MT-MMP, membrane-type MMP; PVDF, poly-(vinylidene difluoride).

Research over the past two decades has clearly established that apolipoprotein E (apoE) prevents vascular disease (*1*, *2*). Apart from its well documented mediation of lipoprotein clearance (*3*), recent evidence suggests that apoE also has direct cell regulatory effects that prevent vascular disease (*4*, *5*). These effects include modulation of an inflammatory response by suppressing lymphocyte activation (*6*, *7*), inhibition of agonist-induced platelet aggregation and activation (*8*, *9*), and suppression of growth factor-induced smooth muscle cell migration and proliferation (*10*, *11*).

Matrix metalloproteinases (MMPs) comprise a family of Zn^{2+}-dependent enzymes that are known to cleave extracellular matrix proteins under both normal and pathological conditions (*12–14*). Currently, 23 MMP genes have been identified in man, and they can be grouped into soluble and membrane-type MMPs (MT-MMPs) (*14*, *15*). Numerous studies have shown that MMPs are expressed during vascular remodeling under pathological conditions, such as those of atherosclerosis, restenosis, and aneurysm formation, but the actual roles of MMPs in the pathogenesises of vascular disease remain unclear (*16*). In atherosclerosis and restenosis, MMPs are produced by the major cell types found in plaques, vessel walls, and leukocytes of the monocyte/macrophage and lymphocytic lineages. A great deal of effort in the field of vascular biology has been centered on the hypothesis that the inhibition of MMP activity may reduce the plaque volume by blocking the migration of smooth muscle cells and macrophages into plaques and thus prevent the later complications of plaque rupture and aneurysm formation. However, the actions of MMPs in complex models of atherosclerosis are largely unknown. A recent study demonstrated that MT1-MMP is expressed by smooth muscle cells and macrophages in human atherosclerotic plaques, and that its expression is up-regulated by proinflammatory molecules (*17*). Thus, activation of smooth muscle cells and macrophages by proinflammatory molecules may influence extracellular matrix remodeling in atherosclerosis by regulating MT1-MMP expression. Although both apoE and MMPs play important roles in vascular disease, their interaction has not been studied so far. In the present study, we found that apoE is a substrate for MT1-MMP, and showed that cleavage of apoE by MT1-MMP abrogates apoE-mediated suppression of cell proliferation. We also discuss the possible physiological significance of degradation of apoE by MT1-MMP.

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MATERIALS AND METHODS

Materials—A human fetal brain cDNA library constructed in the pEAK8 expression vector was obtained from EdgeBio Systems (Gaithersburg, MD). Native apoE protein isolated from human plasma was purchased from Chemicon International Inc. (Temecula, CA). Recombinant MT1-MMP, MMP-7 and MMP-2 catalytic domains tagged with the FLAG epitope at the C terminus were prepared as previously described (*18*, *19*). Monoclonal antibodies directed against the FLAG epitope, glutathione S-transferase (GST), and apoE (IB5-E1) were purchased from Sigma, Santa Cruz Biotech (Santa Cruz, CA), and Progen Biotechnik GmbH (Heidelberg, Germany), respectively.

Cell Culture—Human embryonic kidney HEK293T and human fibrosarcoma HT1080 cells were obtained from the ATCC, and were cultured in DMEM supplemented with 5% fetal calf serum.

Expression Cloning—Expression cloning to identify genes with products that interacted with MMP-2 or MT1-MMP was performed as described previously (*20*).

Construction of an Expression Plasmid for apoE Tagged with FLAG or GST—An expression plasmid for apoE tagged with the FLAG epitope at the C-terminus was constructed by PCR using an apoE reverse primer with an extra *Bgl*II site (underlined) starting at nucleotide 1034 of the apoE gene (GeneBank™ accession number NM_000041) (TGAGATCT GTGATTGTCGCTGGGCACAGGG), as described previously (*20*).

Western Blotting—For Western blot analysis, an apoE (0.4 μg) expression plasmid was co-transfected with either MT1-MMP or a control plasmid (1.6 μg) into HEK293T cells cultured in 35-mm dishes using TransIT LT1 transfection reagent according to the manufacturer's instructions (Mirus, Madison, WI). At 36 h after transfection, the culture medium was replaced with serum-free medium and the cells were incubated for a further 24 h. Cell lysates and protein precipitates obtained from conditioned medium with 10% trichloroacetic acid were analyzed by Western blotting using an anti-apoE antibody as the first antibody, and a goat anti–mouse IgG antibody conjugated with Alexa Fluor 680 (Molecular Probes Inc, Eugenes, OR) as the secondary antibody. The signal was monitored with a LI-COR Odyssey™ infrared imaging system (Lincoln, NE). Precision Plus Protein™ Standards were obtained from Bio-Rad (Hercules, CA).

Preparation of Recombinant apoE-GST Protein (apoE-GST)—An apoE cDNA fragment encoding amino acids 19 to 317 was generated by PCR using a flanking forward primer with an extra *Xho*I site (underlined) starting at nucleotide 138 (AACTCGAGAAGGTGGAGCAAGCGGTGGAGA) and the flanking reverse primer described above. The amplified DNA fragment was digested with *Xho*I and *Bgl*II, and then was inserted into the *Xho*I and *Bgl*II sites of the GST-CTC plasmid as described previously (*21*). Then the apoE GST-fusion protein was purified using GST-Sepharose beads according to the manufacturer's instructions (Amersham Bioscience).

Determination of apoE Protein Cleavage Sites—Recombinant apoE-GST fusion protein (5 μg) was incubated with the recombinant MT1-MMP catalytic domain (0.5 μg) in 30 μl of TNC [50 mM, Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$ (pH 7.4)] buffer containing 0.03% Brij at 37°C for 3 h, and the resulting fragments generated were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto poly-(vinylidene difluoride) (PVDF) membranes (Millipore, Bedford, MA). The N-terminal amino acid sequence of each fragment was determined with a Beckman Coulter LF300 amino acid sequencer.

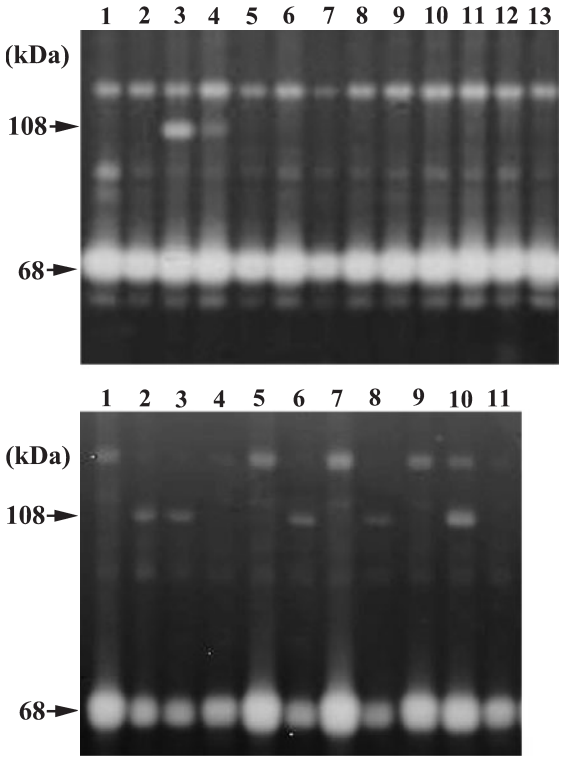

Fig. 1. **Expression cloning. Aliquots of plasmid DNA from the human placental cDNA library were co-transfected into HEK293T cells with MMP-2 and MT1-MMP.** The cells were cultured in 96-well microplates as described under "MATERIALS AND METHODS", and a lysate from each well was subjected to gelatin zymography at 48 h after transfection (upper panel). Note the 108 kDa gelatinolytic band in lane 3. Single clones of plasmid DNA indicated by the first screening were analyzed in the same way (lower panel). The 108 kDa gelatinolytic band can be observed in lanes 2, 3, 6, 8, and 10.

Cell Proliferation Assay—In 96-well tissue culture dishes, 1×10^3 cells per well were incubated for 3 days in DMEM containing 1% fetal calf serum, and then cell proliferation was determined by staining with crystal violet.

RESULTS

Screening of the Human Fetal Brain cDNA Library—After plasmid DNA from the human fetal brain cDNA library had been co-transfected into HEK293T cells with MMP-2 and MT1-MMP cDNAs, cell lysates were analyzed by gelatin zymography. Transfection with MMP-2 and MT1-MMP cDNA generated a 68 kDa gelatinolytic band of latent pro-MMP-2 (Fig. 1). Co-transfection of the cDNA pool generated another gelatinolytic band of 108 kDa in the first screening (upper panel, lane 3). cDNA clones that generated the 108 kDa gelatinolytic band

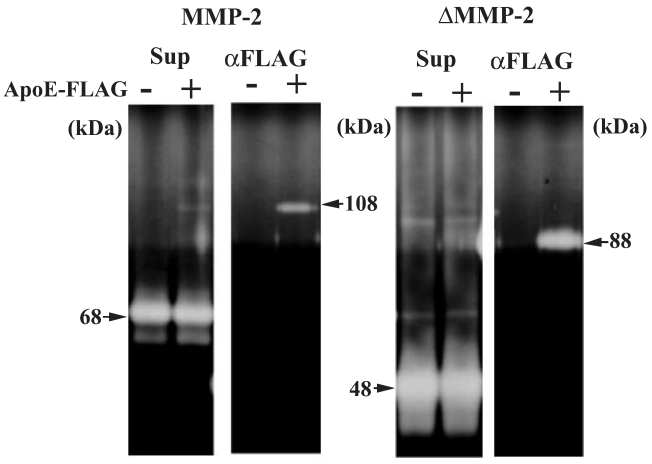

Fig. 2. **Complex formation between pro-MMP-2 and apoE protein.** An expression plasmids for MMP-2 or ΔMMP-2 was transfected into HEK293T cells either alone (lanes –) or with the apoE-FLAG plasmid (lanes +). Culture supernatants were subjected to gelatin zymography (lanes Sup). ApoE-FLAG protein was collected from the culture supernatant of transfected cells using anti-FLAG antibody beads, and co-precipitated MMP was analyzed by gelatin zymography (lanes αFLAG).

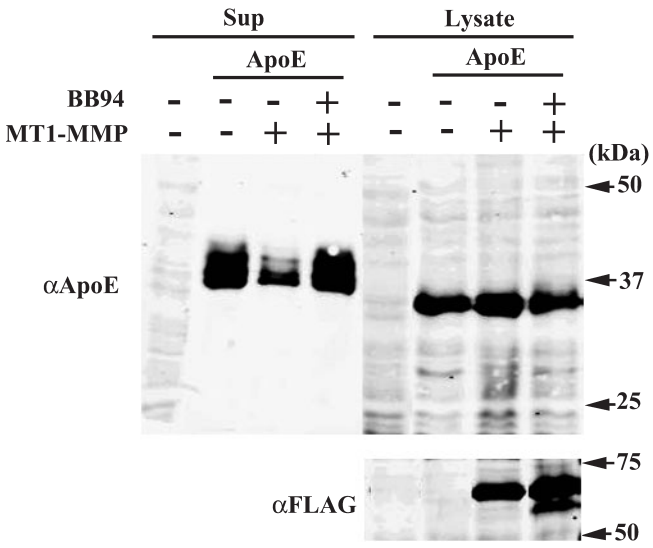

Fig. 3. ***In vivo* degradation of apoE by MT1-MMP.** The expression plasmid for apoE (ApoE) or empty pSG5 plasmid (–) was co-transfected (0.4 μg) with the plasmid for MT1-MMP tagged with the FLAG epitope (1.6 μg) into HEK293T cells cultured in 35-mm dishes. At 36 h after transfection, the culture medium was replaced with serum-free medium with or without 0.1 μM BB94, and the cells were incubated for another 24 h. Culture supernatants (Sup) and cell lysates (Lysate) were then analyzed by Western blotting using anti-apoE antibodies as described under "MATERIALS AND METHODS" (upper panels). Expression of MT1-MMP was examined by Western blotting of cell lysates using anti–FLAG M2 antibodies (lower panel).

were obtained on a second screening (lower panel; lanes 2, 3, 6, 8, and 10), and the nucleotide sequence of the 1.2 kb cDNA fragment was determined. A homology search revealed that this clone was derived from the apoE gene (GeneBank™ accession number NM_000041).

ApoE Protein Forms a Stable Complex with Pro-MMP-2—The apoE gene encodes a 317–amino acid protein with an N-terminal leader sequence of 18 amino acid residues, so the secreted protein comprises 299 amino acid residues. To determine whether the 108 kDa gelatinolytic band detected above represented a complex between apoE protein and pro-MMP-2, apoE protein tagged with the FLAG epitope was co-expressed with MMP-2. Then apoE-FLAG protein collected from the culture supernatant using anti-FLAG antibody beads was analyzed by gelatin zymography (Fig. 2). The 108 kDa gelatinolytic activity was co-precipitated with apoE-FLAG from the culture supernatant of cells transfected with the MMP-2 and apoE-FLAG plasmids, but not from the supernatant of cells transfected with the MMP-2 plasmid alone. An MMP-2 mutant lacking the C-terminal fibronectin- and hemopexin-like domains (ΔMMP-2) was also examined for complex formation with apoE-FLAG, and it was found that ΔMMP-2 was also co-precipitated with apoE-FLAG as an 88 kDa band. These results suggested that pro-MMP-2 forms a stable complex with apoE.

Degradation of apoE by MT1-MMP—We previously demonstrated that KiSS-1 protein, which forms a complex with pro-MMP, serves as a substrate for MMPs (*21*), and this finding led us to examine whether apoE is cleaved by MMPs. After the apoE expression plasmid had been co-transfected into HEK293T cells with either the control or MT1-MMP plasmid, secreted and cell-associated apoE was examined by Western blotting (Fig. 3). ApoE secreted into the culture medium by HEK293T cells transfected with apoE cDNA was detected as a broad band of about 40 kDa, whereas the cell-associated apoE core protein was detected as a 33 kDa band. Co-expression of MT1-MMP reduced the concentration of apoE in the culture medium, but the level of cell-associated apoE core protein was not affected by MT1-MMP expression. Treatment of cells with an MMP inhibitor (BB94) blocked the MT1-MMP-mediated reduction of the apoE level in the culture medium.

To confirm the cleavage of apoE by MT-MMP, the native apoE protein was incubated with recombinant MT1-MMP protein and then analyzed by SDS-PAGE (Fig. 4A). Incubation of apoE protein with MT1-MMP yielded in four major cleavage products. To identify the sites of cleavage, recombinant apoE protein fused to GST was prepared and incubated with MT1-MMP, after which the N-terminal sequences of the cleavage products were determined (Fig. 4B). MT1-MMP cleaved apoE-GST fusion protein at the following peptide bonds; T^{85}-M^{86}, K^{93}-S^{94}, R^{246}-L^{247}, A^{255}-E^{256} and G^{296}-L^{297}. Incubation of apoE-GST fusion protein with MMP-7 resulted in a similar fragmentation pattern with less efficiency (Fig. 4C), but incubation with MMP-2 did not cause any significant cleavage (data not shown).

Effect of apoE on Cell Proliferation—The effect of apoE on cell proliferation was studied with HT1080 cells, which express endogenous MT1-MMP. HT1080 cells stably transfected with the apoE gene (HT/ApoE) secreted a low level of apoE and its fragments (Fig. 5A). Treatment of HT/ApoE cells with BB94 induced accumulation of apoE protein in the culture medium concomitant with suppression of cell proliferation. The effect of apoE on cell proliferation was also compared between HEK293T cells

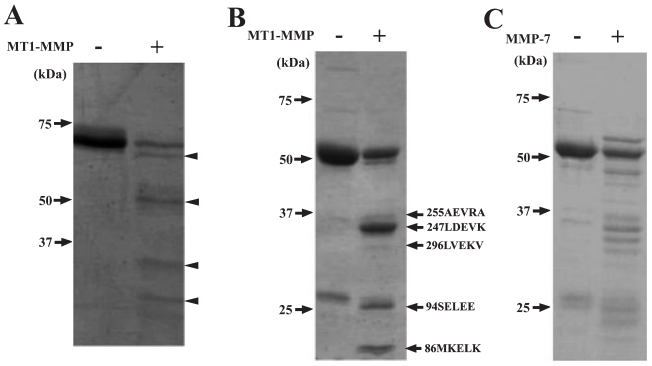

Fig. 4. **Cleavage of apoE by MT1-MMP.** (A) Native apoE protein (5 μg) was incubated with or without the recombinant MT1-MMP catalytic domain (0.5 μg) in 30 μl of TNC buffer containing 0.03% Brij at 37°C for 3 h, separated by 12% SDS-PAGE, and then stained with Coomassie Brilliant Blue. (B) ApoE-GST fusion protein (5 μg) was incubated with recombinant MT1-MMP catalytic domain (0.5 μg), and then analyzed as described above. The N-terminal amino acid sequence of each fragment was determined as described under "MATERIALS AND METHODS." (C) ApoE-GST fusion protein was incubated with recombinant MMP-7, and then analyzed as described above.

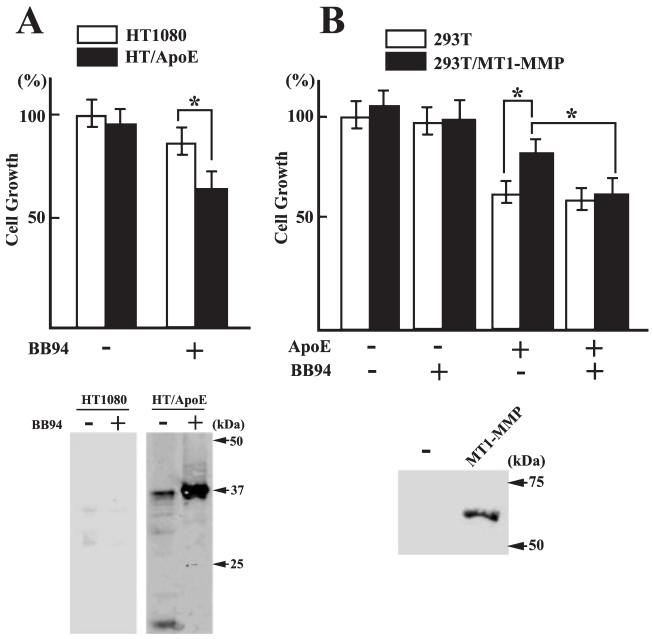

Fig. 5. **Suppression of cell proliferation by apoE.** (A) Proliferation of HT1080 cells transfected with the empty pEAK8 plasmid (HT1080) or apoE plasmid (HT/ApoE) was monitored in the presence or absence of BB94 as described under "MATERIALS AND METHODS" (upper panel). Secretion of apoE protein into the culture medium was detected by Western blotting using anti-apoE antibodies (lower panel). (B) HEK293T cells stably transfected with the empty plasmid (293T) or MT1-MMP plasmid (293T/MT1-MMP) were incubated in the presence or absence of 10 μg/ml apoE-GST protein or 10^{-7} M BB94, and the cell proliferation was determined as described above (upper panel). Expression of MT1-MMP in control HEK293T cells (lane –) or 293T/MT1-MMP (lane MT1-MMP) was detected by Western blotting using anti–MT1-MMP antibodies (lower panel). Bars indicate mean values. *$p < 0.01$.

transfected with a control plasmid and the MT1-MMP plasmid (Fig. 5B). The addition of recombinant apoE-GST protein suppressed proliferation of HEK293T cells transfected with the control plasmid by 35%, whereas that of the cells transfected with the MT1-MMP plasmid was only inhibited by 20%. Simultaneous addition of BB94 with apoE-GST protein significantly suppressed the proliferation of MT1-MMP-expressing cells to the level in the case of control cells treated with apoE.

DISCUSSION

ApoE contributes to protection against vascular disease by mediating lipoprotein clearance and also by regulating various cellular functions, including suppression of growth factor–induced smooth muscle cell migration and proliferation by modulating the inflammatory response. ApoE is known to suppress the proliferation of not only smooth muscle cells but also various other types of cells (*6*). The mechanism for apoE inhibition of cell proliferation is mediated via its binding to cell surface proteoglycans (*22*), and the subsequent activation of inducible nitric-oxide synthase activity (*11*). Some of the functions of apoE may be unrelated to its cholesterol-transporting properties because delipidated apoE remains active in these respects.

The roles of MMPs in the development and progression of atherosclerotic lesions, and in aneurysm formation have only been studied in relation to the degradation of fibrillar collagen. MT1-MMP was identified as an activator of MMP-2 (*21*), and various substrates of MT1-MMP have subsequently been identified, including KiSS-1/metastin, type I collagen, integrin αV, syndecan-1, and interleukin 8 (*23–27*).

In the present study we found that apoE protein forms a stable complex with pro-MMP-2. Previously we reported that KiSS-1 protein, which forms a stable complex with pro-MMP-2, serves as a substrate for MMPs including MMP-2 and MT1-MMP (*23*). Although the physiological significance of the complex formation between pro-MMP-2 and KiSS-1 or apoE remains to be elucidated, the interaction of apoE with MMP led us to examine whether apoE could be a substrate for MMP. The present study showed that apoE protein also serves as a substrate for MT1-MMP and MMP-7, but not for MMP-2. We also showed that the cleavage of apoE by MT1-MMP abrogates suppression of cell proliferation. Recent studies have demonstrated the expression of MT1-MMP by smooth muscle cells and macrophages in atherosclerotic plaques (*17*). Thus, it is possible that the degradation of apoE by MT1-MMP expressed on the surface of smooth muscle cells may down-regulate apoE-mediated suppression of cell migration and proliferation, which may eventually lead to complications such as plaque rupture and aneurysm formation. Better understanding of the role of MT1-MMP in vascular disease may lead to new strategies for the prevention of atherosclerosis.

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