Expression and Localization of Human Endothelin-Converting Enzyme-1 Isoforms in Symptomatic Atherosclerotic Disease and Saphenous Vein

CAROLYN D. JACKSON,*,[1] KAY BARNES,* SHERVANTHI HOMER-VANNIASINKAM,† AND ANTHONY J. TURNER*

*School of Biochemistry & Microbiology, University of Leeds, Leeds LS29JT, United Kingdom; and †Vascular Research Unit, Leeds General Infirmary, Great George Street, Leeds LS13EX, United Kingdom

Endothelin-converting enzyme (ECE-1) is a critical enzyme in the production of the potent vasoconstrictor peptide endothelin (ET-1). It has previously been shown that the levels of both ET-1 and ECE-1 are raised in atherosclerosis, but the possible relevance of the isoforms of ECE-1 in these changes has not yet been investigated. The aim of this study was to examine the expression of the ECE-1a and ECE-1c isoforms in human atherosclerotic pathologies. Immunohistochemical analysis was carried out on sections from atherosclerotic and non-atherosclerotic vascular tissue using a combination of ECE-1 isoform-specific antibodies, anti–α-actin antibodies to identify smooth muscle cells (SMC) and anti-CD68 antibodies to identify macrophages. ECE-1 isoform expression was also examined in cultured SMC and in macrophages isolated from human blood. Results indicated differences in isoform expression in atherosclerotic lesions, with distinct patterns of staining for ECE-1a and ECE-1c. ECE-1c immunoreactivity was seen in macrophages, and also correlated with actin staining. ECE-1a was also localized to macrophages and SMC. Results of this study suggest that these local changes influence the expression patterns of the ECE-1 isoforms within individual cell types. Correlation of these isoform expression patterns with the stage of atherosclerosis could provide novel indicators of disease progression. Exp Biol Med 231:794–801, 2006

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[1] To whom correspondence should be addressed at School of Biochemistry and Microbiology, University of Leeds, Leeds LS29JT, United Kingdom. E-mail: c.d.jackson@bmb.leeds.ac.uk

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Introduction

Endothelin-1 (ET-1), the predominant physiological member of the endothelin family of peptides, was originally identified as a potent vasoconstrictor when it was isolated from the cultured supernatant of porcine aortic endothelial cells (1). Since then, much evidence has accumulated to implicate ET-1 as an important factor in the cardiovascular system, both in the maintenance of basal vascular tone (2) and also in hypertension, chronic heart failure (3), and atherosclerosis (4).

Although predominantly expressed by endothelial cells, ET-1 is also expressed in human macrophages (5) and polymorphonuclear leukocytes (6), suggesting a role for ET-1 in inflammatory processes. ET-1 also acts as a potent mitogen for many cell types, including smooth muscle cells (SMC) and macrophages (7). Atherosclerotic lesions are characterized by neointimal smooth muscle cell proliferation and the infiltration of monocytes, which differentiate into macrophages, the principal inflammatory cells in atherosclerotic plaques. Raised plasma levels of ET-1 in coronary artery disease (8) and the detection of ET-1 in coronary atherosclerotic plaques, where it appears to localize with macrophages and other inflammatory components (9), suggests that ET-1 is involved in the inflammatory processes associated with atherosclerosis.

Endothelin-converting enzyme (ECE-1) is a zinc metalloprotease that catalyses the final step in the biosynthesis of the endothelin family of peptides, cleaving the inactive precursor big-endothelin (big ET-1) to produce the active peptide, endothelin. ECE-1 is a type II membrane protein of which four isoforms have been identified in humans (10). The isoforms differ only in their N-terminal domains and have been designated ECE-1a, -1b, -1c and -1d. The functional significance of the isoforms has not yet been fully elucidated but their different subcellular localizations and promoter sequences suggest potential physiological differences. ECE-1a and ECE-1c are the isoforms

expressed in the cardiovascular system (10), and it has previously been suggested that their expression might be regulated differently under pathological conditions (11, 12). Because the activity of ECE-1 appears to be crucial in the physiological production of ET-1, it is evident that changes in ET-1 levels seen in various pathological conditions may be a reflection of changes in expression or activity of this enzyme or of its isoforms.

There is evidence that increased expression and activity of ECE-1 occur in atherosclerotic arteries compared with histologically normal tissue (13, 14), suggesting a correlation between raised ET-1 levels and ECE-1 in disease. In support of this, ECE-1 colocalizes with actin in SMC (15), and is expressed in both SMC and macrophages in human coronary atherosclerotic lesions (16). Balloon angioplasty–induced neointimal formation in rat, used as a model for human atherosclerosis, has been used to illustrate ECE-1 expression in neointimal SMC and increased expression of both ECE-1 and ET-1 compared to uninjured arteries (16, 17). This suggests that the endothelin system is of significance in neointimal formation and therefore in the progression of atherosclerotic disease. Clinically, raised ET-1 levels are seen following percutaneous transluminal coronary angioplasty, which is used to disperse occlusive atherosclerotic lesions and is frequently complicated by restenosis. It has also been shown that an ET-1 receptor antagonist is vasculoprotective, thus implicating ET-1 in the pathogenesis of angioplasty-induced neointimal formation (18).

Thus, it has previously been shown that the levels of both ET-1 and ECE-1 are raised in atherosclerosis. However, the relevance of the isoforms in these changes has not been elucidated. In this study we have investigated the role of ECE-1 and its isoforms in symptomatic atherosclerotic disease.

Materials and Methods

Antibodies. Isoform-specific antipeptide antibodies were produced as described previously (19). AS65, specific to ECE-1a, and AS66, specific to ECE-1c, were diluted 1/50 and 1/100 for immunostaining. Anti-human α-actin and anti-CD68 antibodies were diluted 1/400 and 1/50 respectively. AEC32–236 to ECE-1 was diluted 1/20 for immunostaining.

Staining of Tissue Sections. Samples of atherosclerotic plaques obtained from patients undergoing carotid and femoral endarterectomies ($n = 10$ patients) and saphenous vein sections obtained from patients undergoing bypass surgery for symptomatic peripheral atherosclerotic disease ($n = 3$ patients) were immersed in formalin before paraffin embedding and sectioning. Sections were subsequently stained according to Barnes *et al*. (20). The investigation conforms with the principles outlined in the Declaration of Helsinki (Cardiovascular Research 1997;35: 2–3).

Isolation of Monocytes. Monocytes were isolated from buffy coats (National Blood Service, Leeds, United Kingdom) as described previously (21). Purified monocytes were differentiated into macrophages by culturing in RPMIpen/strep with 10% human serum (v/v) for 9–13 days. Macrophages were converted into foam cells by incubation with 50 μg/ml acetylated low-density lipoproteins (LDL) for 4 days.

Culture of Human Aortic Smooth Muscle Cells (HaSMC). HaSMC were grown in medium containing supplements according to suppliers' instructions (Promocell, Heidelberg, Germany).

Immunofluorescence. Immunofluorescence of cultured HaSMC and macrophages was carried out as previously described (22). The cells were fixed by the addition of ice-cold methanol/acetone (1:1).

RNA Preparation and Reverse Transcription–Polymerase Chain Reaction (RT-PCR). Tissue samples were crushed to a fine powder under liquid nitrogen with a pestle and mortar before RNA extraction. RNA was prepared from both tissue samples and cultured cells using Trizol reagent according to manufacturer's instructions. RT-PCR was carried out using the Enhanced Avian RT-PCR kit in a two-step reaction. For primers and PCR conditions, see Table 1.

Results

The focus of this study is the expression of the isoforms ECE-1a and ECE-1c in symptomatic atherosclerotic disease. Sequential sections from atherosclerotic plaques were stained with elastic van Gieson stain to visualize the overall pathology of the specimens. Macrophages and SMC were identified with anti-CD68 and anti–α-actin antibodies respectively. ECE-1 expression was determined using isoform-specific antibodies (19).

ECE-1a and ECE-1c Are Localized to Macrophages. The elastic van Gieson stain revealed that all the 10 plaques examined were of an advanced nature with a necrotic core and fibrous cap identifiable in each case (Fig. 1A). Macrophage-rich regions were also visible with this

Table 1. Primer Sequences and Conditions Used for Amplification of ECE-1a (AFOR, AREV) and ECE-1c (CFOR, CREV) in RT-PCR Reactions

Primer	Sequence	Expected size (bp)	Conditions
AFOR	ATGCCTCTCCAGGGCCTGGGC	108	95°C 180 secs, 65°C 90 secs, 72°C 30 secs (30 cycles)
AREV	AGTTCACCTGCAGGGAAGGAG		95°C 180 secs, 65°C 90 secs, 72°C 30 secs (30 cycles)
CFOR	GGAGCACGCGAGCTATGATG	715	94°C 30 secs, 56.4°C 45 secs, 72°C 1 min (35 cycles)
CREV	CTGTTGGAGTTCTTGGAATC		94°C 30 secs, 56.4°C 45 secs, 72°C 1 min (35 cycles)

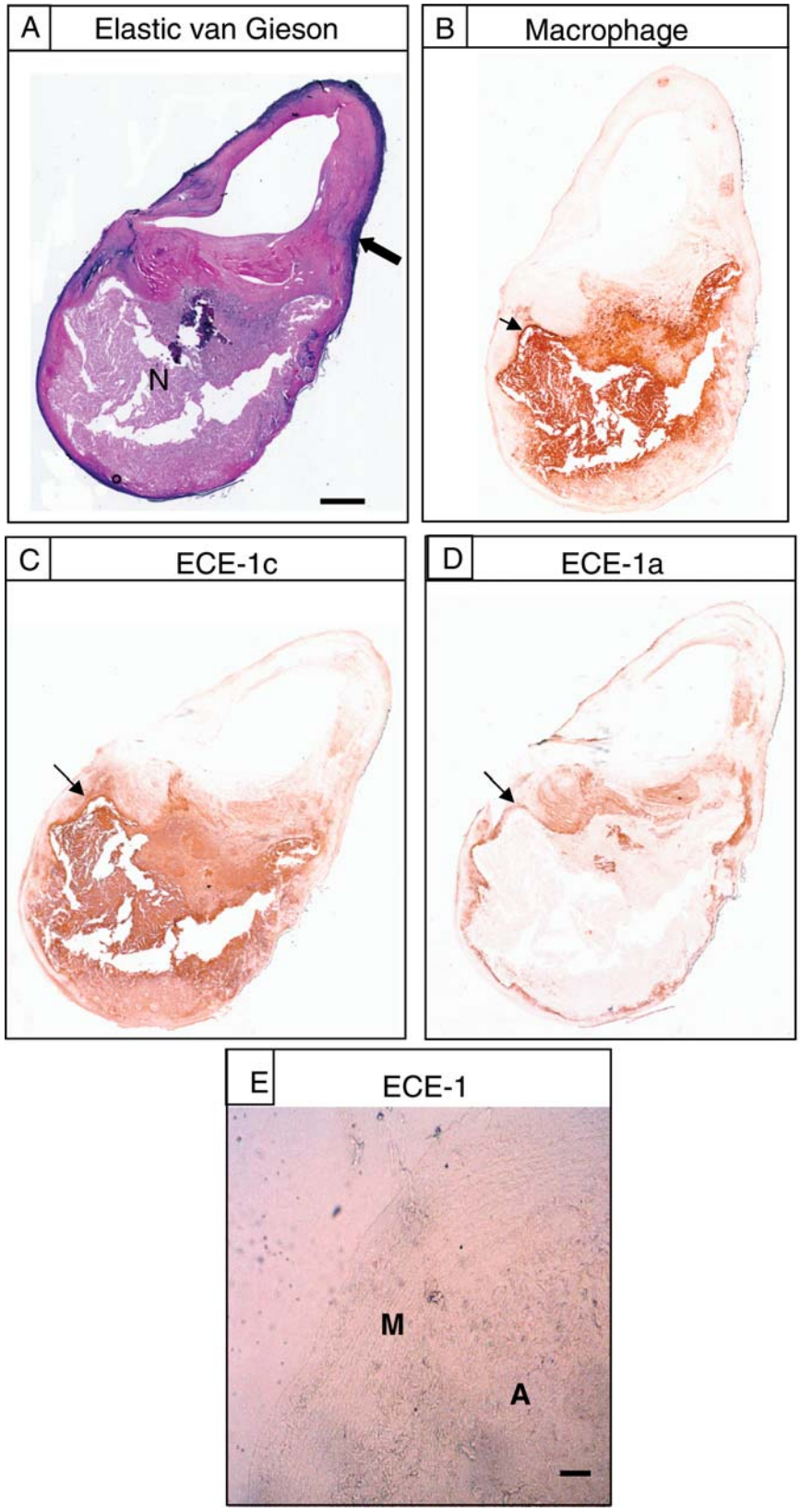

Figure 1. Serial sections of a representative atherosclerotic tissue specimen. Sections are stained with elastic van Gieson stain (A), anti-CD68 for macrophages (B), AS66 for ECE-1c (C), and AS65 for ECE-1a (D). N represents the necrotic core, the large arrow in (A) indicates elastin and in (B) the arrow head indicates a macrophage-rich area. The small arrows in (C) and (D) indicate macrophages immunostaining for ECE-1c and ECE-1a, respectively. There was complete loss of immunoreactivity of these two isoforms when the primary antibodies were replaced with either preimmune serum or rabbit IgGs (results not shown). An additional control section (E) was taken from a saphenous vein and immunostained with AEC32-236, a monclonal antibody that recognizes all isoforms of ECE-1. No staining for ECE-1c or ECE-1a is present in the smooth muscle tissues of the tunica media (M) or in the large muscular veins of the broad tunica adventitia (A). Bars, 100 μM (A–D); 30 μM (E). (Color figures available in on-line version.)

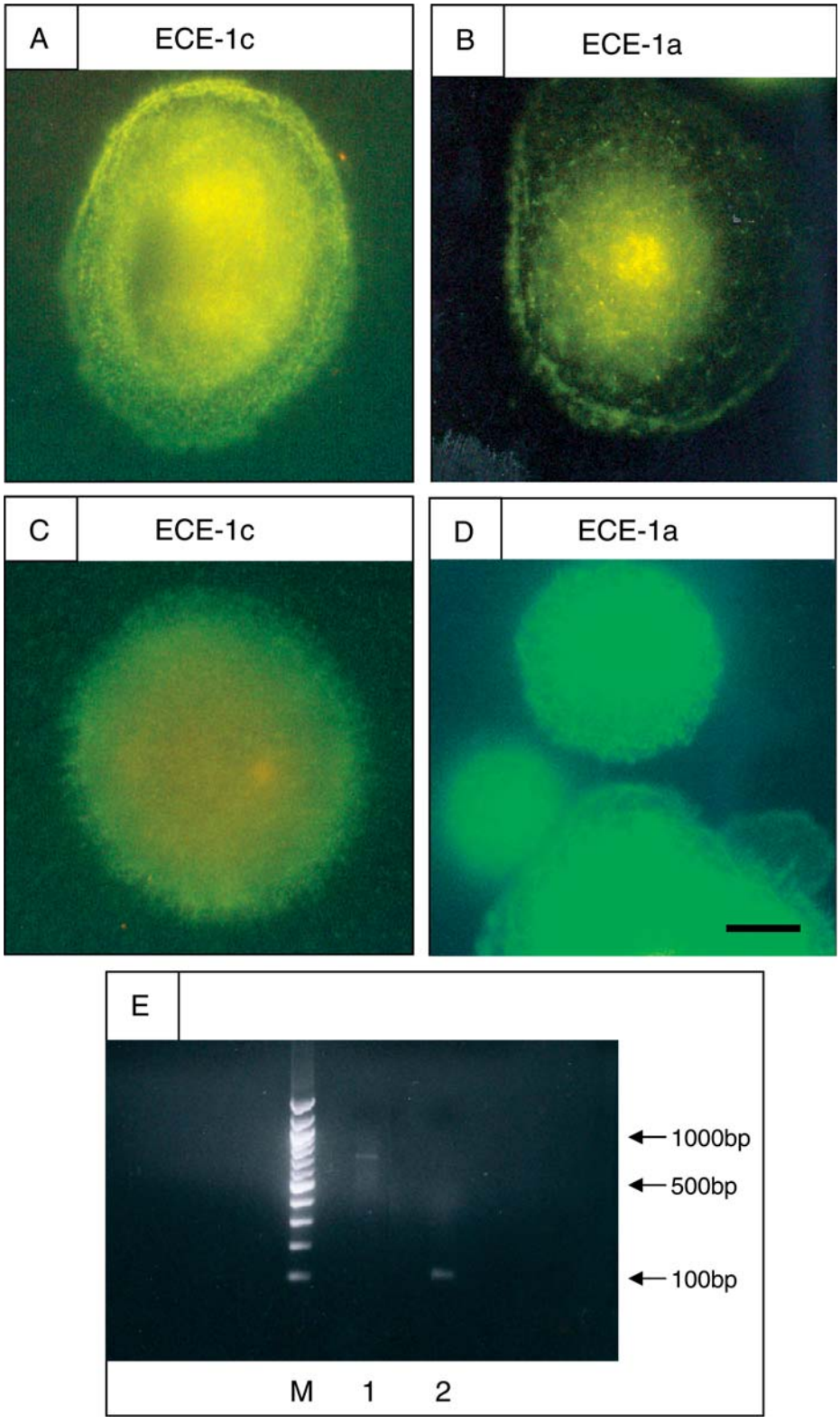

Figure 2. Representative cells illustrating typical ECE-1c and ECE-1a expression patterns in monocyte-derived human macrophages. Untreated (A and B) and acetylated LDL-treated (C and D) cells are immunostained for ECE-1c (A and C) and ECE-1a (B and D). RT-PCR (E) reveals expression of ECE-1c (Lane 1) and ECE-1a (Lane 2) in these cells. Bar, 10 μM (A–D). (Color figures available in on-line version.)

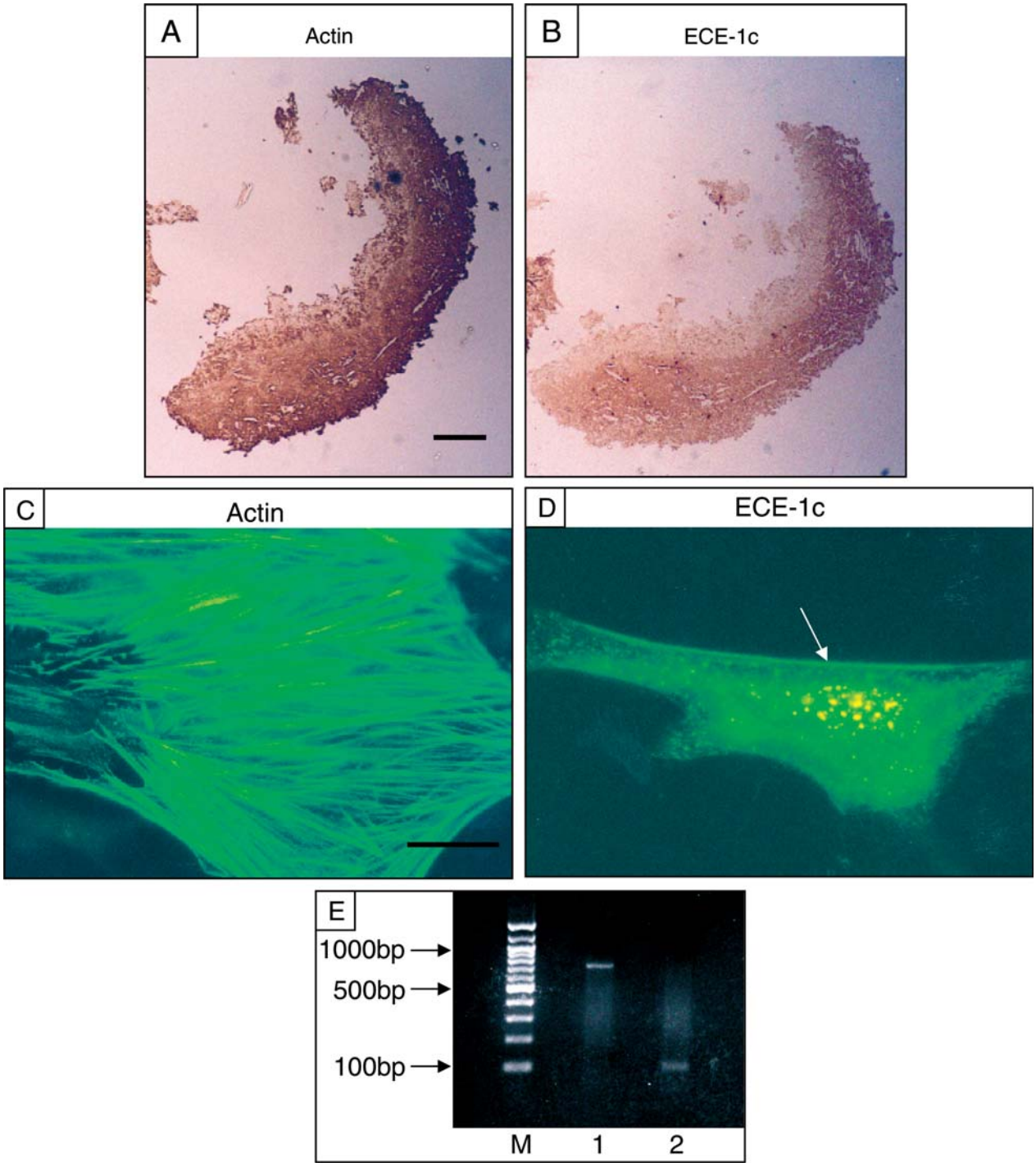

Figure 3. Isoform expression in serial sections from a representative carotid plaque specimen and human smooth muscle cells. Immunohistochemical staining for SMC actin (A) and ECE-1c (B) in carotid plaque. ECE-1c immunoreactivity colocalizes with SMC actin. In human aortic SMC, actin (C) and ECE-1c (D) expression is revealed by immunofluorescence (arrow denotes plasma membrane ECE-1c). RT-PCR reveals expression of ECE-1c (Lane 1) and ECE-1a (Lane 2) in these cells (E). Bars, 50 μM (A and B); 10 μM (C and D). (Color figures available in on-line version.)

stain and were further identified with anti-human macrophage antibody (Fig. 1B). All 10 of the 10 carotid plaque samples examined showed immunoreactivity for ECE-1c and ECE-1a. Figures 1C and D, respectively, illustrate the staining patterns characteristic of each isoform. In necrotic areas of the plaque where there is an accumulation of injured or dead cells, residual ECE-1c and macrophage staining remained. No immunoreactive staining was present when the primary antibodies were replaced either with preimmune rabbit or purified rabbit IgGs (results not shown). Although the main purpose of the study was to examine expression of the isoforms in diseased tissues, we

also applied a monoclonal antibody, AEC32-236, that recognizes both the isoforms of ECE-1 to asymptomatic saphenous vein tissue. In three examples of this healthy tissue with intact vessel walls, no ECE-1 (Fig. 1E) or macrophage staining (not shown) was detected.

Following the localization of the ECE-1 isoforms to macrophages in tissue sections, their expression in these cells was examined in culture. Macrophages isolated as monocytes from human blood and differentiated in culture expressed both ECE-1c and ECE-1a, as illustrated by immunostaining (Fig. 2A and B, respectively). ECE-1a is expressed at low levels in comparison with ECE-1c in these cells. Following treatment with acetylated LDL to convert macrophages into foam cells, immunofluorescence studies revealed an apparent increase in expression of ECE-1a (Fig. 2D). In contrast, ECE-1c appeared to be expressed more weakly after treatment (Fig. 2C). The presence of both these isoforms of ECE-1 was confirmed in macrophages by RT-PCR (Fig. 2E).

ECE-1a and ECE-1c Are Localized to Smooth Muscle Cells. Immunoreactive ECE-1c was present in regions of the plaque that were also immunostaining for actin (Fig. 3B and A, respectively). Cultured human aortic SMC were probed with the isoform antibodies. ECE-1c was abundantly expressed in a punctate fashion (Fig. 3D) in the actin-positive muscle cells (Fig. 3C). RT-PCR confirmed the expression of ECE-1c in these cells (Fig. 3E). The weaker expression of the ECE-1a RNA in SMCs was reflected by very weak immunostaining (not shown) of the cultured cells and plaques (not shown).

ECE-1a and ECE-1c Are Expressed in Atherosclerotic Plaques. To demonstrate the presence of ECE-1c and ECE-1a in plaques, RT-PCR of total RNA isolated from the samples was performed (Fig. 4A and B, respectively). Variable amounts of ECE-1a and ECE-1c RNA were detected in samples taken from different plaques. In particular, in two samples (Fig. 4, Lanes 1 and 3), there appeared to be exceptionally high expression levels of ECE-1a RNA (above the levels of ECE-1c RNA) compared with the sample in Figure 4, Lane 4. Interestingly, in this study, the LDL-treated macrophages also showed higher ECE-1a than ECE-1c expression (Fig. 2D and C, respectively). The transformed endothelial cell line Eahy926 (23), which expresses both ECE-1a and ECE-1c, was used as a positive control.

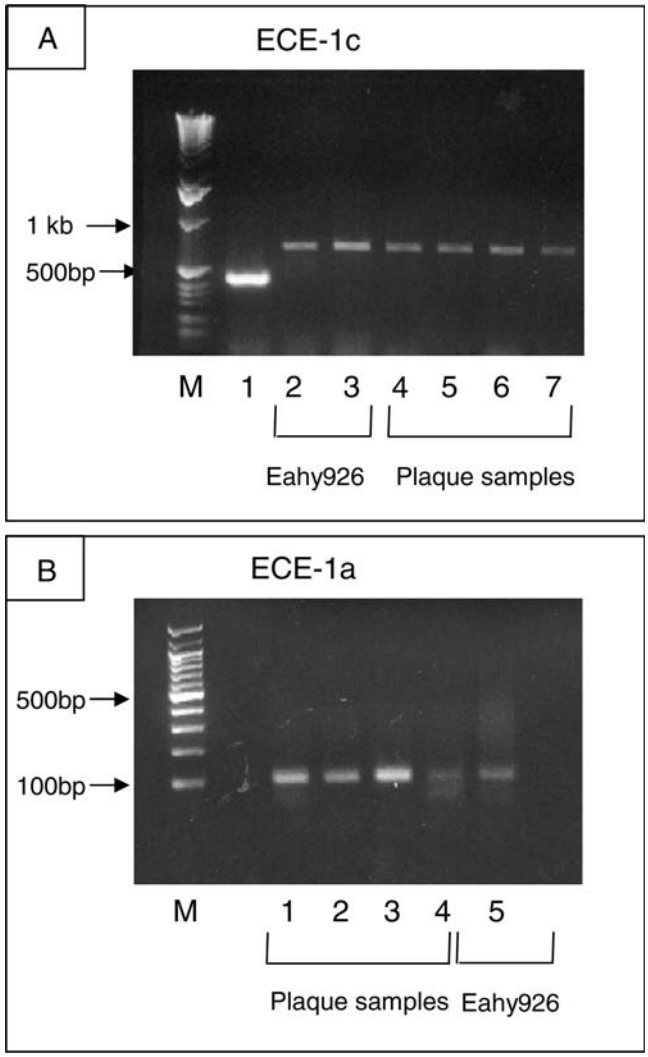

Figure 4. Expression of ECE-1c (A) and ECE-1a (B) expression in atherosclerotic tissue samples. In (A), Lane 1 is a 444-bp control; Lanes 2 and 3 are controls showing ECE-1c expression in Eahy926 cells, which are known to express ECE-1a and c isoforms; and Lanes 4 to 7 show analysis of atherosclerotic samples from different patients. In (B), Lanes 1–4 show ECE-1a expression in atherosclerotic samples from different patients and Lane 5 shows ECE-1a expression in Eahy926 cells.

Discussion

In this study we compare ECE-1a and ECE-1c isoform expression in pathological tissues and cultured SMC and macrophages by using immunocytochemistry and RT-PCR. Examination of sections of atherosclerotic lesions commonly revealed ECE-1c and ECE-1a in macrophages and in smooth muscle cells. Previously, it has been shown that ECE-1 colocalizes with actin filaments in rat and human smooth muscle cells (15). In this study we have illustrated that it is the ECE-1c isoform that is predominantly present in the smooth muscle cells of atherosclerotic plaque and in cultured human smooth muscle cells.

In atherosclerosis, recruitment of monocytes to the vessel wall and their subsequent differentiation into macrophages and uptake of LDL play a crucial part in the development of the atherosclerotic lesion. The accumulated macrophages constitutively secrete a variety of cytokines and growth factors into the local environment, which stimulate local medial SMC to undergo accelerated proliferation and differentiation. Investigation of the mechanism behind these effects has revealed 40 genes with altered expression in human SMC treated with the conditioned medium of activated macrophages (24).

Changes in gene expression are also seen in macrophages undergoing transformation to the foam cell phenotype (25). Therefore, locally acting factors, mediating cross talk between several cellular components, may influence gene expression in SMC and macrophages within atherosclerotic lesions, which then contribute to disease progression.

Investigation of macrophages and macrophage-derived foam cells in culture indicated that changes in ECE-1 isoform expression occur following acetylated LDL treatment. Increased expression and changes in distribution of ECE-1a were seen, concomitant with decreased ECE-1c expression, as the macrophages became foam cells. These results indicate that locally acting factors influence ECE-1 isoform expression in macrophages and may account for the variable levels of total RNA isoform expression in the patient samples assayed and the changes in ET-1 levels in atherosclerosis recorded by other authors (9). In addition, recent findings suggest that administration of big ET-1 results in enhanced vasoconstriction and increased formation of ET-1 in patients with atherosclerosis (26). Indeed, a recent study indicated that lipid-loading of macrophages, to establish a foam cell–like phenotype, affected gene expression and lipopolysaccharide-induced nuclear factor-κB (NF-κB) activation (25). Binding sites for NFκB are present in the promoter regions of the ECE-1a and ECE-1c gene (27), therefore implicating NF-κB as a potential regulatory factor in ECE-1a expression in atherosclerosis.

It is therefore evident that macrophages, SMC, and their local environments undergo changes with disease progression in atherosclerosis. The combined data from this study indicate that changes in ECE-1 isoform expression occur in macrophages both in tissue sections and in culture at different stages. These differing ECE-1 isoform expression levels may be directly responsible for the increased levels of mature ET-1 found in atherosclerotic pathologies and may therefore contribute directly to the pathophysiology of the disease. It is also possible, therefore, that these changes correlate with disease progression and clinical presentation, and may be indicative of plaque stability, stage of disease, and prognosis. Further investigation to determine the mechanisms, and the transcription factors, behind these changes would be beneficial in defining the roles of the ECE-1 isoforms in symptomatic atherosclerotic disease.

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