Epitope-specific antibody-induced cleavage of angiotensin-converting enzyme from the cell surface

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Angiotensin I-converting enzyme (ACE; CD143, EC 3.4.15.1) is a type-1 integral membrane protein that can also be released into extracellular fluids (such as plasma, and seminal and cerebrospinal fluids) as a soluble enzyme following cleavage mediated by an unidentified protease(s), referred to as ACE secretase, in a process known as 'shedding'. The effects of monoclonal antibodies (mAbs) to eight different epitopes on the N-terminal domain of ACE on shedding was investigated using Chinese hamster ovary cells (CHO cells) expressing an ACE transgene and using human umbilical vein endothelial cells. Antibody-induced shedding of ACE was strongly epitope-specific: most of the antibodies increased the shedding by 20–40%, mAbs 9B9 and 3A5 increased the shedding by 270 and 410% respectively, whereas binding of mAb 3G8 decreased ACE shedding by 36%. The ACE released following mAb treatment lacked a hydrophobic transmembrane domain anchor. The antibody-induced

shedding was completely inhibited at 4 °C and by zinc chelation using 1,10-phenanthroline, suggesting involvement of a metalloprotease in this process. A hydroxamate-based metalloprotease inhibitor (batimastat, BB-94) was 15 times more efficacious in inhibiting mAb-induced ACE shedding than basal (constitutive) ACE release. Treatment of CHO-ACE cells with BB-94 more effectively prevented elevation in antibody-dependent (but not basal) ACE release induced by 3,4-dichloroisocoumarin and iodoacetamide. These data suggest that different secretases might be responsible for ACE release under basal compared with antibody-induced shedding. Further experiments with more than 40 protease inhibitors suggest that calpains, furin and the proteasome may participate in this process.

Key words: ACE, monoclonal antibody, proteolytic cleavage, secretase, shedding.

INTRODUCTION

Angiotensin I-converting enzyme (ACE; kininase II, CD143, EC 3.4.15.1) is a Zn²⁺ carboxypeptidase that cleaves two vasoactive peptides, angiotensin I and bradykinin. This enzyme plays an important role in blood-pressure regulation, the development of vascular pathology and endothelium remodelling in some disease states [1,2]. The major tissue isoform of ACE has two highly similar domains (N and C domains), each bearing a functional catalytic site [3]. ACE is a type-1 integral membrane protein that is anchored to the plasma membrane near its C-terminus. Somatic ACE also exists as a soluble form (e.g. in plasma, amniotic and cerebrospinal fluids and seminal plasma [1,2]). Plasma ACE is probably derived from vascular endothelial cells [4] and lacks a transmembrane portion at the C-terminus [5].

Significant progress has been made in the study of ACE release from the cell surface. ACE secretase was shown to be a metalloprotease [5,6] that co-localized with ACE in a number of tissues, including pig kidney, lung and testis, and human lung and placenta. However, its activity was absent from human kidney and pig small-intestinal brush-border membranes [6]. ACE secretase has an absolute requirement for its substrate, ACE, to be anchored in a membrane for cleavage to occur [7,8]. This is a phenomenon that applies to several other secretases, including those responsible for the cleavage of amyloid precursor protein, Kit ligand and colony-stimulating factor (for reviews, see [9,10]).

Several cell models have been used to study the release of ACE: rabbit, human and Chinese hamster ovary (CHO) cell lines transfected with cDNAs coding for rabbit or human testicular, or human somatic ACE [11–13]. ACE secretase activity (measured as the appearance of soluble ACE) was inhibited successfully by different hydroxamic acid-based compounds [such as batimastat (BB-94)], with IC₅₀ values in the low micromolar range [8,13]. Experiments with somatic ACE and mutants of the stalk regions of ACE molecule in different cell models have provided some information on the mechanism of ACE cleavage by ACE secretase [13–15]. These data indicate that the ACE secretase in CHO cells is not constrained by a particular cleavage site but rather is influenced by the proximity of the extracellular domain to the membrane. Moreover, the C-terminal region of the protein contains a recognition motif that is obscured by the N-terminal domain of somatic ACE [7,15,16].

ACE is a member of the growing family of membrane proteins that are cleaved proteolytically in the juxtamembrane stalk by a proteolytic activity now referred to as a secretase or sheddase [9,10]. The ACE secretase appears to be similar to, but not identical with, tumour necrosis factor- α secretase and amyloid precursor protein α -secretase [10,17,18]. Nevertheless, despite considerable effort in this field, the mechanism of production of soluble ACE *in vivo*, the components involved in this process and the factors that regulate generation of soluble ACE have not been delineated specifically. Little is known about the effects of

Abbreviations used: ACE, angiotensin I-converting enzyme; mAb, monoclonal antibody; CHO, Chinese hamster ovary; HUVEC, human umbilical vein endothelial cell; BB-94, batimastat; DCI, 3,4-dichloroisocoumarin; Tos-Phe-CH₂Cl, N-tosyl-L-phenylalanylchloromethane; IA, iodoacetamide; DTT, dithiothreitol; LDH, lactate dehydrogenase; PKC, protein kinase C; ZIE, benzyloxycarbonyl-Ile-Glu(OtBu)Ala-Leu-aldehyde; Dec-RVKR-cmk, decanoyl-Arg-Val-Lys-Arg-chloromethylketone; Hip-His-Leu, hippuryl-His-Leu.

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Figure 1 mAb-induced ACE shedding

CHO-ACE cells were incubated with different anti-ACE mAbs (10 μ g/ml) at 4 °C in a binding assay (**A**, cell ELISA) and at 37 °C in an assay of ACE release (**B**) for 4 h. (**C**) The ratio of shedding to binding for each mAb. * $P < 0.05$, $n = 3$. The horizontal line on (**A**) is the background for mAb binding with non-specific, non-immune mouse IgG (mlgG). The line on (**B**) is the basal shedding in the presence of non-immune mouse IgG.

antibody binding on ACE release. An understanding of the fine mechanism of the interaction of anti-ACE antibodies with the cell surface and the physiological effects of this interaction remains to be elucidated. In the present study the development of a convenient cell model for the study of ACE release [12,19] in combination with the availability of a unique set of monoclonal antibodies (mAbs) directed to eight different epitopes of human ACE [20] allowed us to demonstrate epitope-dependent antibody-induced proteolytic cleavage and release of membrane-bound ACE. Different patterns of inhibition of basal and antibody-induced ACE shedding by numerous inhibitors of proteases of different classes suggested the presence of two different secretases for ACE release into the circulation under normal and 'antibody-induced' conditions.

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MATERIALS AND METHODS

Materials

Cell-culture reagents and lipofectin were purchased from Gibco BRL Life Technologies (Grand Island, NY, U.S.A.); antibiotics and anti-mycotics were from Sigma (St. Louis, MO, U.S.A.). BB-94 was synthesized at SmithKline Beecham Pharmaceuticals (Harlow, Essex, UK). PMA, dithiothreitol (DTT), calcium ionophore A23187, ionomycin, E64, E64c, E64d, α_2 -microglobulin, phosphoramidon, 3,4-dichloroisocoumarin (DCI), calpain inhibitors I and II, trypsin inhibitor, chymostatin, leupeptin, pepstatin, PMSF, *N*-tosyl-L-phenylalanylchloromethane (Tos-Phe-CH₂Cl, 'TPCK'), *N*-tosyl-L-lysylchloromethane ('TLCK'), dithionitrobenzoate, p-hydroxymercuriphenylsulphonate, *N*-ethylmaleimide, iodoacetamide (IA) and iodoacetic acid were purchased from Sigma. Calpeptin and benzyloxycarbonyl-Val-Ala-Asp(OMe)-CH₂F (ZVAD-fmk) were purchased from Calbiochem-Novabiochem AG (La Jolla, CA, U.S.A.). SJ6017A was from Senju Pharmaceutical Co. (Kobe, Japan). Benzyloxycarbonyl-Ile-Glu(otBu)Ala-Leu-aldehyde (ZIE; where otBu means t-butyl ester), decanoyl-Arg-Val-Lys-Arg-chloromethylketone (Dec-RVKR-cmk), acetyl-Trp-Glu-His-Asp-aldehyde (Ac-WEHD-cho), acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-cho) and acetyl-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVAD-cmk) were purchased from Bachem Bioscience (King of Prussia, PA, U.S.A.). JTP-4819 was from Japan Tobacco (Osaka, Japan).

CFP-pAB was provided kindly by Dr I. Smith (Baker Medical Research Institute, Prahran, Australia), 9-fluorenyl-methoxycarbonyl-prolyl-pyrrolidine-2-nitrile (Fmoc-Pro-Pyrr-CN) by Dr S. Wilk (University of New York, New York, NY, U.S.A.), benzyloxycarbonyl-propyl-3-fluoropyrrolidine (Z-Pro-Pyrr) by Dr S. Scharpe (University of Antwerp, Wilrijk, Belgium), compound 68 by Dr G. de Nanteuil (Servier, Suresnes, France), P18 by Dr G. Thomas (Oregon University, Portland, OR, U.S.A.) and lactacystine by Dr S. Omura (The Kitasato Institute, Tokyo, Japan).

Cell culture

Human umbilical vein endothelial cells (HUVECs; second or third passages) were plated on to gelatin-coated plastic and cultured, as described previously [21], in Medium 199 supplemented with 200 μ g/ml endothelial cell growth supplement obtained from human brain [22]. A stable line of CHO cells (2C2 clone), expressing wild-type human ACE was obtained and cultured as described previously [19].

ACE shedding assay

Cells growing in 96-well microtitre plates were washed three times with Hanks balanced salt solution and incubated with inhibitors diluted in serum-free Complete Culture Medium (Mediatech, Herndon, VA, U.S.A.) for at least 30 min prior to addition of mAbs (10 μ g/ml), also diluted in Complete Culture Medium. After 4 h, the culture medium was collected, while cells were washed several times and fixed with 4% paraformaldehyde for subsequent quantitation of bound mAbs in cell ELISA assays. To determine cell-associated ACE activity as well as to quantify correctly the rate of ACE release (by referring the ACE level in culture medium to that on the cell surface), cells were lysed with 100 μ l of 8 mM CHAPS. Both supernatants

Figure 2 Time- and dose-dependence of antibody-induced shedding of ACE from CHO-ACE cells

(A) CHO-ACE cells were incubated with mAbs 9B9, 3A5, i2H5 and non-immune mouse IgG (mIgG; as a control) at 10 μ g/ml for 24 h. At each time point culture medium was collected and stored at 4 °C until assay for ACE activity ($n = 3$). (B) CHO-ACE cells were incubated with mAbs at the indicated concentrations for 4 h. ACE activity in the culture medium was measured using Hip-His-Leu as a substrate. Data presented as means $\pm$ S.D. ($n = 3$).

and lysates were centrifuged and ACE activity was determined using a fluorimetric assay. Aliquots (10–50 μ l) were added to 200 μ l of 5 mM hippuryl-His-Leu (Hip-His-Leu; substrate for ACE) and incubated for the appropriate time at 37 °C. The reaction was terminated with 0.28 M NaOH, and the His-Leu product was estimated fluorimetrically [23].

Cell ELISA

CHO cells expressing somatic ACE (clone 2C2; CHO-ACE cells) [19] or HUVECs (third passage) [22], cultured as described previously, were grown in a 96-well microtitre plates. Cells were chilled on ice for at least 30 min and washed several times with cold PBS. Control mouse IgG or 10 μ g/ml anti-ACE mAbs in PBS/casein (0.2%) were added and incubated for 2 h on ice. After washing, cells were fixed with 4% paraformaldehyde for 15 min at room temperature, washed several times (with PBS) and bound mAbs (which reflect membrane-bound ACE) were quantified by incubation with anti-mouse antibody conjugated with alkaline phosphatase, followed by spectrophotometric assay [21].

Phase separation

The separation of ACE from cell lysate and culture medium into aqueous and detergent phases was performed according to Bordier's method of separation of integral membrane proteins [24]. Briefly, an aliquot of cell lysate or culture medium was prepared in 200 μ l of 10 mM Tris/HCl, pH 7.4, containing 150 mM NaCl and 1% Triton X-114, at 0 °C. This sample was overlaid on 300 μ l of a 6% sucrose solution in 10 mM Tris/HCl, pH 7.4, containing 150 mM NaCl, and incubated at 37 °C for 5–10 min before being centrifuged at 3000 g for 3 min. The 200 μ l of upper aqueous phase was collected and 50 μ l of ice-cold 2.5% Triton X-114 was added to a final concentration of 0.5%. After chilling to 0 °C, the mixture was overlaid repeatedly on the same cushion of sucrose solution and incubated and centrifuged as described above. At the end of separation, the aqueous and detergent phases were collected, adjusted to the same final

concentration of Triton X-114 and 10 μ l of each was taken for measurement of ACE activity with 2 mM benzyloxycarbonyl-Phe-His-Leu or with 5 mM Hip-His-Leu as a substrates for ACE, as above.

Cytotoxicity assay

All inhibitors were tested for cytotoxicity on CHO-ACE cells using a lactate dehydrogenase (LDH) assay kit from Promega (Madison, WI, U.S.A.) according to the manufacturer's recommendations. Briefly, cells were incubated with different concentrations of inhibitors for 4 h. Then 50 μ l aliquots from wells were transferred to a fresh 96-well plate and 50 μ l of reconstituted substrate was added to each well and incubated for 30 min in the dark. Stop solution (50 μ l) was added and the absorbance was recorded at 490 nm. Bovine heart LDH was included as a positive control. To determine total LDH levels, cells were lysed by adding a 10-fold volume of lysis solution (from the LDH assay kit) before being assayed as described above.

Other methods and statistical analysis

The plate-precipitation assay for estimation of ACE activity in the presence of ACE inhibitors and Western blotting with anti-ACE antibodies were performed as described previously [20,25].

Data are represented as means $\pm$ S.D. Groups were compared using a two-tailed Student's t test.

RESULTS

mAb-induced ACE release

As we described previously [19], CHO cells transfected with somatic ACE (CHO-ACE cells) demonstrated a low level of spontaneous ACE release from the cell surface into the culture medium over a 24 h period, corresponding to 10–20% of the total ACE (released plus cell-associated). Stimulation of CHO-ACE cells with PMA enhanced ACE shedding by up to 165% over a 4 h period compared with spontaneous release. This

Figure 3 Effect of low temperature and 1,10-phenanthroline on basal and antibody-induced ACE shedding

(A) CHO-ACE cells were incubated in the presence of mAbs to ACE (10 μ g/ml) at 4 or 37 $^{\circ}$ C for 4 h ($n = 3$). (B) CHO-ACE cells were pre-incubated with 1,10-phenanthroline (5 mM) during 30 min at 37 $^{\circ}$ C, after which mAbs to ACE (10 μ g/ml) were added for the subsequent 4 h. ACE release was estimated by measuring ACE activity in the culture fluid ($n = 3$). (C) The mAb binding in the presence of 1,10-phenanthroline was estimated by cell ELISA, as described in the Materials and methods section ($n = 3$). mlgG, non-immune mouse IgG.

demonstrated that ACE shedding could be modulated in this cell system, which as such might prove a relevant model for the effects of other agents [19].

We studied the effects of mAbs directed to eight different epitopes on the extracellular N-terminal domain of ACE [20] on their ability to induce ACE shedding from the cell surface. Interaction of ACE with two mAbs, 9B9 and 3A5, caused a significant increase in ACE activity in culture medium: approx. 2.7- and 4.1-fold greater than the control, respectively (Figure 1B). This effect was reproduced in cultured HUVECs, where the

same two mAbs, 9B9 and 3A5, out of the eight anti-ACE mAbs studied, increased ACE shedding by 1.4- and 2-fold, respectively (results not shown). It is important to note that one mAb, 3G8, significantly decreased ACE shedding to 64% of the control values for basal shedding (see the Discussion).

The increased ACE shedding induced by mAbs 9B9 and 3A5 was not caused by the increased binding of these two mAbs to the surface of the cells compared with the other mAbs. mAb binding to cells was measured using a cell ELISA on cells at 4 $^{\circ}$ C (to avoid internalization). Accordingly, the extent of binding of 9B9 and 3A5 was comparable with other mAbs (Figure 1A). We calculated the ratio of ACE shedding to the extent of mAb binding in order to express the ability of the mAbs to induce ACE shedding from CHO-ACE cells. mAbs 9B9 and 3A5 have 6- and 17-fold, respectively, greater propensities to induce ACE shedding compared with other antibodies (Figure 1C). These results suggest that the shedding of ACE from the plasma membrane of CHO-ACE cells induced by mAbs is epitope-specific. The interaction of mAbs 9B9 and 3A5 may alter the conformation of the extracellular domain of ACE and thereby render the protein more susceptible to cleavage by ACE secretase.

Characterization of antibody-induced shedding of ACE

mAb-induced ACE shedding was detectable within 30 min and gradually increased during a 24 h period of incubation (Figure 2A). There was no difference in ACE shedding mediated by mAb 9B9 within the concentration range of 3–30 μ g/ml (Figure 2B). However, ACE shedding mediated by mAb 3A5 reached an effect ceiling at 10 μ g/ml. In all following experiments we used mAbs at a concentration of 10 μ g/ml.

To determine whether antibody-induced shedding of ACE is dependent on secretase activity, CHO-ACE cells were pre-chilled for 30 min on ice and incubated with 10 μ g/ml mAbs 9B9 and 3A5 at 37 and 4 $^{\circ}$ C for 4 h. Under these conditions, both basal and antibody-induced shedding of ACE was completely blocked at low temperature (Figure 3A). Figure 3(B) shows that 1,10-phenanthroline, a zinc chelator that inhibits all zinc metalloproteases, completely prevented shedding to the basal level and only slightly inhibited non-stimulated, basal shedding. The 1,10-phenanthroline itself did not affect the binding of mAbs to CHO-ACE cells (Figure 3C). These results suggest that the secretase involved in antibody-induced shedding is a zinc metalloprotease.

To identify whether ACE released into the culture medium by mAbs 9B9 and 3A5 is soluble and lacks a transmembrane anchor, we performed Triton X-114 phase-separation experiments. ACE released to the culture medium collected from the cells incubated with mAbs 9B9 and 3A5, and that released under basal conditions, is concentrated in the aqueous phase after phase separation and therefore lacks a transmembrane domain (results not shown).

Inhibition of antibody-induced ACE shedding by BB-94

BB-94, a zinc metalloprotease inhibitor, was used to characterize further the secretase(s) involved in antibody-induced ACE shedding. BB-94 inhibits matrix metalloproteinases, the α -secretase involved in cleaving the amyloid precursor protein and tumour necrosis factor- α secretase [10,17,18]. In our assay the efficacy of BB-94 for the inhibition of antibody-induced shedding was 15-fold higher than for the inhibition of basal (constitutive) ACE shedding (Figure 4). BB-94 did not inhibit ACE catalytic activity at the concentrations used, up to 100 μ M, and therefore the reduction of ACE activity in the culture medium of CHO-ACE can be ascribed to an inhibition of ACE shedding. This is the first

Figure 4 Effect of BB-94 on basal and antibody-induced ACE shedding

CHO-ACE cells were preincubated with BB-94 at the indicated concentrations for 30 min at 37 °C. Then mAb 3A5 was added (10 µg/ml) and cells were incubated for an additional 4 h at 37 °C. ACE release was estimated by measurement of ACE activity in the culture fluid.

indication that the secretase involved in antibody-induced ACE shedding differs from that responsible for the basal shedding of ACE from the cell membrane.

Regulation of ACE release

To characterize further the secretase activities involved in basal and antibody-induced ACE release from the cell membrane, we tested the effects of a range of enzyme inhibitors directed against different mechanistic classes of proteases (Table 1). The effect of these inhibitors on ACE release was examined by their inclusion in the culture medium. None of the inhibitors used was toxic at the concentrations used, as determined by the LDH assay (results not shown).

The release of ACE was not affected by inhibitors of endoproteases, neutral endopeptidase, aspartic proteases and most of the serine-protease inhibitors. However, PMSF, Tos-Phe-CH₂Cl and DCI dramatically induced ACE shedding, especially in the presence of mAb 3A5. A similar effect with DCI on basal ACE release was demonstrated using CHO cells transfected with human testicular ACE [26,27], but not with renal tubular epithelial cells transfected with rabbit testicular ACE [13]. The thiol reagent IA, but not iodoacetic acid, at 30 µM increased ACE release, especially under antibody-induced conditions. The reducing agent, DTT, abolished both basal and 3A5-induced shedding. As DTT (as well as DCI) reversibly inhibits ACE activity, the ability of these compounds to affect ACE shedding was quantified by plate-precipitation assay and Western-blot analysis (results not shown). ACE release was also elevated by treating cells with the calcium ionophores A23178 and ionomycin (Table 1).

None of the inhibitors tested (see Tables 1 and 2) influenced the stability of soluble ACE in the culture medium (results not shown). Therefore, the increase in the levels of ACE observed in

culture medium in the presence of certain inhibitors (DCI, Tos-Phe-CH₂Cl and IA) was due to an effect on ACE shedding rather than a due to the inhibition of other proteases that might hypothetically degrade ACE.

We also estimated the effect of tested inhibitors on cell-associated ACE during the shedding procedure (4 h). After collecting the culture medium, washed cells were fixed and the level of membrane-bound ACE was estimated by cell ELISA. In the case of basal shedding experiments, we incubated fixed cells with mAb I2H5. In those experiments in which antibody-induced shedding was performed, cells were already pre-incubated with mAb 3A5, and therefore the amount of bound mAb 3A5 reflected the level of ACE remaining on the cell surface.

We demonstrated a significant increase in basal ACE shedding, i.e. the appearance of higher ACE activity in culture medium (as was the case with DCI, Tos-Phe-CH₂Cl and IA) that was accompanied by a small (5–14%), albeit consistent decrease in cell-associated ACE level (estimated via binding of mAb I2H5). In the case of 3A5-induced shedding (when ACE activity in culture fluids increased 2–3-fold) the disappearance of cell-associated ACE in the presence of DCI, Tos-Phe-CH₂Cl and IA was much more pronounced (results not shown). We also confirmed this observation by assaying cell-bound ACE activity (results not shown). Therefore, we can conclude that several inhibitors stimulated the release of somatic ACE into the cell medium with a concomitant decrease in membrane fraction.

Effect of kinase inhibitors on PMA and antibody-induced shedding

As we and others have shown previously, PMA stimulates ACE release [21,26,28,29]. In this study we examined the effects of two different kinase inhibitors on basal, PMA- and antibody-induced ACE shedding. CHO-ACE-cells were pre-incubated with staurosporine, a protein kinase C (PKC) inhibitor, and genistein, an

Table 1 Effect of protease inhibitors on basal and antibody-induced ACE shedding

CHO-ACE cells were pre-incubated with inhibitors at the indicated concentrations for 30 min at 37 °C. Then mAbs to ACE or control non-immune mouse IgG (mlgG) were added (10 µg/ml) and incubated for an additional 4 h at 37 °C. The effects of the compounds were expressed as a percentage of ACE activity/concentration level in the vehicle control cultures. The S.D. in control groups (performed separately for each compound) did not exceed 10%. **P* < 0.05 compared with the control. Tos-Lys-CH₂Cl, *N*-tosyl-L-lysylchloromethane pHMS, *p*-hydroxymercuriphenylsulphonate.

Protease	Inhibitor	Concentration	ACE shedding (%)	
			+ Control mlgG	+ mAb 3A5
Aspartate	Pepstatin	10 µg/ml	99.5 ± 5.3	119.8 ± 2.7
Neutral	Phosphoramidon	100 µM	88.0 ± 5.6	93.4 ± 2.7
endopeptidase				
Endoproteinase	α ₂ -Macroglobulin	10 µg/ml	116.9 ± 14.9	85.5 ± 20.1
Serine	PMSF	1 mM	157.0 ± 14.8*	154.7 ± 2.6*
	DCI	100 µM	147.5 ± 5.3*	326.4 ± 24.6*
	Tos-Phe-CH ₂ Cl	100 µM	262.3 ± 15.7*	410.8 ± 29.8*
	Tos-Lys-CH ₂ Cl	100 µM	110.5 ± 8.6	131.1 ± 3.9*
	Leupeptin	100 µM	93.3 ± 12.2	114.9 ± 0.8*
	Chymostatin	100 µM	124.1 ± 26.8	85.3 ± 9.4
	Trypsin inhibitor	10 µg/ml	103.1 ± 11.8	95.8 ± 4.1
Cysteine (thiol reagents)	E64	30 µM	102.3 ± 11.6	97.2 ± 1.2
	Dithionitrobenzoate	100 µM	112.7 ± 6.1	104.3 ± 2.8
	pHMS	30 µM	149.3 ± 13.3*	154.8 ± 5.0*
	<i>N</i> -ethylmaleimide	10 µM	106.4 ± 19.4	131.6 ± 10.3*
	IA	30 µM	167.4 ± 8.7*	326.6 ± 16.2*
	Iodoacetic acid	30 µM	151.6 ± 18.2*	97.2 ± 5.6
	DTT	1 mM	34.6 ± 3.2*	36.6 ± 6.2*
Ca ²⁺ effectors	Ionomycin + Ca ²⁺	10 µg/ml	169.4 ± 13.5*	186.1 ± 5.8*
	A23178	1 µM	220.6 ± 26.3*	169.0 ± 8.9*

Figure 5 Effect of staurosporine on basal and PMA- and antibody-induced ACE shedding

CHO-ACE cells were pre-incubated with staurosporine at 100 nM for 30 min at 37 °C. Then PMA (100 nM) or mAbs (10 µg/ml) were added for the next 4 h at 37 °C. ACE release was estimated by measuring ACE activity in the culture fluid. **P* < 0.05, statistically significant differences within the pairs of bars indicated. mlgG, non-immune mouse IgG.

inhibitor of tyrosine kinase phosphorylation. ACE release was then estimated under basal conditions or in the presence of PMA as well as mAb 3A5. Genistein at 100 µM did not inhibit ACE

Table 2 Effect of protease inhibitors on basal and antibody-induced ACE shedding

Details are as for Table 1. **P* < 0.05, compared with the control. ZVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp(OMe)-CH₂F; Ac-WEHD-cho, acetyl-Trp-Glu-His-Asp-aldehyde; Ac-DEVD-cho, acetyl-Asp-Glu-Val-Asp-aldehyde; Ac-YVAD-cmk, acetyl-Tyr-Val-Ala-Asp-chloromethylketone; Fmoc-Pro-Pyrr-CN, 9-fluorenyl-methyloxycarbonyl-propyl-pyrrolidine-2-nitrile; Z-Pro-Pyrr, benzyloxycarbonyl-propyl-3-fluoropyrrolidine; mlgG, non-immune mouse IgG.

Protease	Inhibitor	Concentration	ACE shedding (%)	
			+ Control mlgG	+ mAb 3A5
Neutral endopeptidase (EC 3.4.24.15)	CFP-pAB†	100 µM	112.1 ± 8.9	90.45 ± 7.8
Prolyl endopeptidase	Fmoc-Pro-Pyrr-CN	100 µM	113.1 ± 6.4	89.6 ± 9.2
	JTP-4819	100 µM	110.6 ± 4.8	99.6 ± 5.1
	Z-Pro-Pyrr	100 µM	96.0 ± 6.2	106.9 ± 5.6
	Compound 68	100 µM	116.0 ± 26.3	77.0 ± 6.52
Caspase	ZVAD-fmk	100 µM	63.7 ± 10.8	85.2 ± 4.51
Caspase I	Ac-YVAD-cmk	100 µM	107.9 ± 4.3	95.0 ± 10.7
	Ac-WEHD-cho	100 µM	99.0 ± 15.1	85.9 ± 16.2
Caspase III	Ac-DEVD-cho	100 µM	88.1 ± 19.3	76.2 ± 5.4*
Calpain	E64	30 µM	102.3 ± 11.6	97.2 ± 1.2
	E64c	100 µM	105.9 ± 12.3	93.1 ± 1.8
	E64d	100 µM	98.0 ± 9.0	108.6 ± 1.8
	Calpain Inhibitor I	100 µM	153.3 ± 3.1*	238.9 ± 18.9*
	Calpain Inhibitor II	100 µM	99.5 ± 5.7	103.6 ± 5.6
	Calpeptin	62.5 µM	228.6 ± 14.5*	295.6 ± 21.5*
	SJ6017A	30 µM	101.2 ± 8.6	97.4 ± 5.1
	ZIE	30 µM	126.2 ± 23.4	220.3 ± 34.6*
Furin	Dec-RVKR-cmk‡	300 uM	178 ± 25*	308 ± 45*
	PI8	45 µg/ml (1 µM)	109.3 ± 6.2	86.8 ± 6.4*
Proteasome	Lactacystin	30 uM	115.9 ± 5.3	170 ± 10.4*

† In the presence of 10 µM phosphoramidon.

‡ ACE shedding was quantified by ACE ELISA because the furin inhibitor demonstrated strong inhibition of His-His-Leu hydrolysis by the C domain of ACE (S. M. Danilov and I. V. Balyasnikova, unpublished work).

Figure 6 Effect of membrane antigen cross-linking by mAbs on ACE cleavage

HUVECs (third passage) were incubated overnight with 15 mAbs (10 μ g/ml) to different endothelial antigens (from the sixth Human Leucocyte Differentiation Antigens workshop [32a]), and then ACE activity in the culture fluid was estimated enzymically (see the Materials and methods section). Raw data (absorbance values) for 14 (out of 15 mAbs that were tested) are not shown because they did not induce ACE shedding. mAbs 9B9 and 3A5 were used as positive controls for antibody-induced ACE shedding. * $P < 0.05$, differences are statistically significant in comparison with control non-immune mouse IgG (mlgG).

shedding under any conditions (results not shown). As expected, PMA-induced shedding of ACE was inhibited to basal levels by staurosporine at 100 nM, whereas ACE shedding under basal conditions was not affected. However, an opposite effect on antibody-induced shedding was demonstrated with staurosporine; this compound increased mAb-induced ACE shedding (Figure 5) further. This result implies that the signalling mechanisms involved in antibody-induced shedding may be different from those that operate in constitutive or PMA-induced ACE release.

Effect of protease inhibitors on antibody-induced ACE shedding

Analysis of the increased release of ACE from CHO-ACE cells caused by class-specific protease inhibitors (Table 1) showed that the protease(s) (or other elements of the biochemical events leading to ACE release) that increase ACE shedding are (i) chymotrypsin-like, (ii) thiol-sensitive and (iii) Ca^{2+} -induced. At least five classes of proteases have similar properties (based on inhibitor sensitivity): neutral metalloproteases, prolyl endopeptidases, caspases, calpains and furin, as well as other members of the family of proprotein convertases. We examined the effect of

a number of more selective inhibitors on these classes of protease (Table 2). Several (but not all) inhibitors of calpains, as well as a furin inhibitor enhanced the rate of ACE release by 2–3-fold compared with the control. As in the case of less specific protease inhibitors (Table 1), the effect of these inhibitors on the antibody-induced shedding of ACE was more prominent than on the basal ACE release.

Effect of endothelial antigen cross-linking by mAbs on ACE shedding

There are numerous examples of cross-linking of membrane antigens by mAbs dramatically inducing proteolytic cleavage (shedding) of these antigens from the cell surface (for review, see [30]). Moreover, there are several examples showing that cross-linking of one antigen results in shedding of another antigen; e.g. cross-linking of CD95 induced shedding of CD44 [31], cross-linking of thrombomodulin caused shedding of ACE, and vice versa [32].

In order to explore whether the effects of antibody-induced ACE shedding may have physiological relevance, we tested the ability of mAbs to endothelial antigens to induce ACE shedding from the surface of HUVECs. In the first series of experiments we tested mAbs directed to 15 different endothelial antigens (from the collection of the Endothelial Section of the sixth Human Leucocyte Differentiation Antigens workshop [32a]). One of these mAbs (anti-E-selectin) significantly induced ACE shedding (Figure 6). We then tested serum of patients with systemic lupus erythematosus, which contain different anti-endothelial antibodies [33], for the ability to shed ACE after binding with the surface of HUVECs. From 20 sera tested (compared with eight control normal sera), three caused a statistically significant increase in ACE shedding (results not shown).

DISCUSSION

We investigated the biochemical mechanisms involved in antibody-induced shedding of ACE from the cell surface of CHO-ACE cells. As we described previously, CHO-ACE cells demonstrate a spontaneous release of ACE from the plasma membrane with a rate similar to that shown with endothelial cells [19]. We have found that treatment of CHO-ACE cells with anti-ACE mAbs can markedly up-regulate the release into the culture medium of wild-type somatic ACE. We observed a similar effect using cultured HUVECs. These data suggest that the mechanism of shedding induced by anti-ACE mAbs is common to CHO-ACE cells and HUVECs, thus validating our use of the CHO-ACE cell-culture paradigm to explore this phenomenon.

Anti-ACE mAb-induced shedding is an epitope-dependent process because only two mAbs (9B9 and 3A5) from eight assayed, all recognizing different epitopes on ACE molecule, induced ACE shedding (Figure 1). The constitutive release of ACE is mediated by proteolytic cleavage of the stalk sequence adjacent to the membrane anchor [7]. We think that binding of mAbs 9B9 and 3A5 alters the conformation of the large extracellular domain of ACE, thus exposing the stalk region and facilitating access of the secretase(s) involved in this process (see the scheme in Figure 7). Antibody-induced internalization is unlikely to be a relevant mechanism for ACE release, as 9B9 and 3A5 do not differ from other anti-ACE mAbs in this regard [19].

We demonstrated previously that the mAb 3A5, which exerted the strongest effect on ACE shedding, is also the mAb whose binding profoundly changed the conformation of ACE. Thus binding of this mAb significantly inhibits catalytic activity of ACE (despite the antibody's epitope being distant from the

Figure 7 Hypothetical scheme of the regulation of ACE release

(A) Under basal conditions, release of native ACE from the cell surface is regulated by ACE secretase. (B) Binding of some anti-ACE mAbs (as well as different mutations in the stalk region of ACE) [34–36] dramatically alters the conformation of the ACE globule, thus favouring the exposition of the stalk region of ACE from the cell surface even more effectively than normal ACE secretase under basal conditions.

active-site cleft). Also, binding of this mAb to ACE prevents binding of other mAbs directed at the N-terminal fragment, also suggesting a significant change in ACE conformation [20]. Therefore we can consider that the ligation with mAbs such as 3A5 (and perhaps 9B9) rendered the ACE protein more susceptible to proteolysis. These two mAbs recognized different, but overlapping, epitopes on ACE [20]. Recently we have found that the epitopes for mAbs 9B9 and 3A5 are localized between Lys-535 and Lys-572 on the N domain of ACE (I. V. Balyasnikova, V. G. Gavriluk, Y. E. Eliseeva, E. V. Kugaevskaya and S. M. Danilov, unpublished work). The reason why the conformation of ACE is so sensitive to mAb binding to this particular region of the N domain remains to be elucidated.

Further support for the notion that the conformation of the ACE protein markedly affects its susceptibility to cleavage came from two recently published papers studying the effect of mutations in the stalk region of ACE on the rate of ACE shedding. We demonstrated that the mutation of Pro-1199 → Leu in the stalk region of human somatic ACE leads to a 5-fold increase of ACE shedding [34]. This increase is in the same order of magnitude as 3A5 binding, and this accelerated ACE solubilization was inhibited by metalloprotease inhibitor compound 3 [35]. However, another mutation in the stalk region of the testicular ACE, Asn-631 → Gln, which corresponds to Asn-1196 in somatic ACE, leads to much more pronounced increase in shedding: after 4 h of chase the majority of mutant ACE was secreted from the cell and was no longer present in the cell lysate [36]. Interestingly, the cleavage of this mutant ACE was not blocked by the metalloprotease inhibitor BB-94 but by the serine-protease inhibitor 1,3-dichloroisocoumarin. Such a mutation may permit the action of other distinct proteases.

We speculate that a local conformational modification caused by mutations in a stalk region of ACE [34–36] or by binding with

mAb 3A5, which leads unambiguously to gross conformational changes in the ACE molecule [20], results in better accessibility of the stalked region to another secretase on the cell membrane (Figure 7).

The fact that mAb 3G8 inhibited proteolytic cleavage of ACE from the cell surface is especially interesting. Recently it was shown that somatic ACE can form dimers in artificial membrane preparations (reverse micelles) and this process was inhibited effectively by several carbohydrates, including galactose [37]. We have found that, among our set of anti-ACE antibodies, only two mAbs, which are directed to overlapping but not identical epitopes, 3G8 and 9B9 [20], inhibited dimer formation in micelles. Moreover, in the presence of galactose, 9B9-induced ACE shedding was abolished completely (O. A. Kost, E. E. Chemo-danova, I. V. Balyasnikova, I. I. Nikolskaya and S. M. Danilov, unpublished work). Therefore, we suggest that the mechanism of ACE shedding might depend on the dimerization status of ACE on the cell membrane.

Since treatment of the cells with PMA, an activator of PKC, accelerates ACE shedding, we suggest that PKC might be involved also in antibody-induced shedding of ACE. Surprisingly, staurosporine, a PKC inhibitor, which abolished PMA-induced shedding of ACE to basal levels, but did not affect spontaneous shedding, promoted an additional increase in 3A5- and 9B9-induced shedding. This suggests that the regulatory mechanisms for constitutive, PMA- and antibody-induced shedding are different and involve PKC activity in PMA- and antibody-induced shedding. Our data are in agreement with Borrego et al. [38] and Harrison et al. [39], who demonstrated the involvement of PKC in PMA-induced shedding of CD16 from natural killer cells. The authors proposed a different mechanism of shedding, independent of PKC and induced by cross-linking of CD16-II using mAbs [38]. Recently, the interaction of PKC

and ACE during PMA-induced ACE cleavage was demonstrated [29].

However, neither PMA- nor antibody-induced shedding of ACE is dependent on the activation of protein tyrosine kinase, since it was not affected by genistein, a specific inhibitor of protein tyrosine kinase. Similar data were obtained for CD16-II of natural killer cells cross-linked with mAbs [38].

The secretase activity involved in constitutive ACE shedding belongs to the metalloproteinase class of enzymes [6,8,13], which co-localize with ACE in a number of tissues, including pig kidney, lung and testis and human placenta and lung, although the activity was absent from human kidney and small-intestinal brush-border membranes [6]. The rate of ACE release from the cell surface of different ACE-expressing cells was inhibited successfully by different hydroxamic acid-containing compounds (like BB-94), with IC_{50} values in the micromolar range in cell-free systems [8,13]. In our experiments BB-94 was a potent inhibitor of constitutive ACE release (Figure 4), in full agreement with other studies [6,8,13]; however, the inhibition of antibody-induced shedding was 15-fold more efficient. These data suggest that different or additional proteolytic activities operate in constitutive compared with antibody-induced ACE release.

Similarly, 1,10-phenanthroline, a zinc chelator that inhibits all metalloproteinases, further distinguished between two proteolytic activities involved in spontaneous and antibody-induced shedding. 1,10-Phenanthroline completely inhibited antibody-induced ACE release but had only a modest effect on basal ACE shedding (Figure 3). We should note that the effect of 1,10-phenanthroline as well as of BB-94 and other metalloproteinase inhibitors on the inhibition of the basal shedding on intact cells (this study and [13,27,35,36]) was not complete, in contrast with almost total inhibition of ACE shedding by these compounds in cell-free conditions [6]. This may be due to restricted access of the inhibitors to proteases in intact cells, because the efficacy of the inhibition dramatically increased in the membrane fractions that have been prewashed with detergent [40]. Another possible reason for non-complete inhibition of ACE shedding by BB-94 is so-called 'burst' of ACE release. The process of washing and refeeding of the cells expressing ACE resulted in a transient stimulation (or burst) of the rate of ACE release [26,27], which might not have been mediated by the 'classical' ACE secretase. We found that the proportion of BB-94-inhibited ACE release into the culture fluid increased with the duration of time after of washing and refeeding (results not shown).

We used different proteinase inhibitors to characterize the class of proteinase involved in ACE release induced by antibodies (Table 1). Among serine-protease inhibitors, PMSF, Tos-Phe-CH₂Cl and DCI all had the unexpected effect of increasing ACE release. Moreover, the effect of DCI and Tos-Phe-CH₂Cl was more pronounced on antibody-induced shedding of ACE than on basal release (Table 1). The diverse effects of these inhibitors on basal and antibody-induced shedding further supports the idea that different proteolytic processes are involved in each circumstance. Others have reported similar data with DCI on the constitutive release of ACE [26,27]. It is possible that the secretase(s) participating in both constitutive and antibody-induced shedding can be degraded constitutively by proteases sensitive to DCI, thus decreasing the activity of ACE secretase. However, recent data indicate that serine-protease inhibitors may affect the trafficking of substrate proteins to the site of secretase(s) action rather than the release process itself [10,41].

The activity of secretase(s), responsible for the basal and antibody-induced ACE shedding, or some other part of the biochemical pathway can be regulated by Ca²⁺ effectors, since both A23178 and ionomycin enhanced ACE release to the same

extent (Table 1). Our data agree with reports showing the activation of solubilizing proteases in response to calcium influx [42]. As ACE shedding can be modulated by intracellular calcium levels and thiol redox reagents, it is conceivable that calpains may play a role in this process. In our study, the calpain inhibitor I, calpeptin and ZIE increased both basal and, to a greater extent, antibody-induced ACE shedding (Table 2). Although other inhibitors of calpains did not effect ACE release from the cell surface, it is possible that the secretase cleaving ACE can itself be degraded by calpains. However, at present it is not possible to make definitive statements regarding the role of calpain in the absence of specific inhibitors.

To investigate the role of the proteasome in ACE release, we treated cells with lactacystin, a specific proteasome inhibitor [43,44]. Lactacystin is most potent against the chymotrypsin-like activity of the proteasome. Antibody-induced shedding was increased in the presence of lactacystin (Table 2), thus implicating proteasomes in the cascade of events involving ACE release from the cell surface.

An increase in both spontaneous and, to a greater extent, antibody-induced shedding of ACE was also shown with Dec-RVKR-cmk, a specific inhibitor of furin (Table 2). Among the known substrates for furin are some membrane-bound glycoproteins such as the insulin-receptor precursor, the soluble precursor of von Willebrand factor, the activin A and B precursor polypeptide, β -nerve growth factor and complement pro-C3 (for review, see [45]).

Stimulation of different surface receptors with mAbs trigger the cleavage of these receptors, leading to their down-regulation and the release of their soluble extracellular domains from the cell surface into the medium (for review, see [30]). It was demonstrated previously that binding of polyclonal anti-ACE antibodies induced ACE antigenic modulation, leading to ACE shedding from the luminal surface of the endothelium (for review, see [46]). We have shown that both binding of mAb 9B9 to endothelium in culture and its specific pulmonary accumulation, as a result of binding with lung endothelium *in vivo*, enhances ACE shedding from the endothelium [47].

Whereas numerous publications have focused on the identification of different anti-endothelial autoantibodies, there are very few reports of anti-ACE autoantibodies. When we developed an assay for the determination of ACE levels in human blood (ACE ELISA), we found that blood from a small number of normal volunteers (three out of 138) probably contained anti-idiotypic (anti-9B9) antibodies [25]. The presence of anti-idiotypic antibodies implies the existence of anti-ACE antibodies. Recently, the presence of autoantibodies to ACE was indicated in some patients with arterial hypertension and some autoimmune diseases [48]. Therefore, for a limited number of patients, it is possible that anti-ACE antibodies may cause cross-linking of ACE on the surface of the endothelial cells. Shedding of cell-surface proteins can be induced by antibodies raised to different cell-surface proteins. Thus antibody directed to rabbit thrombomodulin caused the rapid redistribution and shedding of ACE into circulation *in vivo*. At the same time, thrombomodulin release was augmented by anti-ACE antibodies [32]. Recently, auto-antibodies directed against thrombomodulin were found in patients with a history of thrombotic complications [49].

Therefore, at least two antibodies, anti-thrombomodulin [32] and anti-E-selectin (Figure 6), effectively down-regulate ACE expression on the cell surface. Thus this mechanism represents a novel pathway for inducing ACE cleavage.

Information about the mechanism of ACE release might also have practical significance. The association of one of the polymorphic variants of the ACE gene (DD genotype, characterized

by elevated ACE levels in blood and tissue) with a higher frequency of myocardial infarction, diabetic nephropathy, stroke and hypertension, and the beneficial therapeutic effects of ACE inhibitors indicate that elevated ACE levels can be a new risk factor in some cardiovascular and renal diseases [50,51]. In contrast, higher levels of ACE expression might be beneficial for preventing the development of Alzheimer's disease [52]. However, the prognostic value of the determination of ACE activity in the blood is limited without information about the mechanisms of release of ACE from the tissue (mainly endothelial cells) to the circulation, hence the need to understand ACE secretase and its regulation.

In conclusion, we found that anti-ACE antibodies dramatically increased ACE shedding from the surface of CHO-ACE cells in an epitope-dependent manner. The secretase involved in antibody-induced ACE release can be distinguished from that responsible for constitutive ACE shedding. Both constitutive and antibody-induced ACE shedding are complex processes, potentially involving different proteases. Further study is necessary to identify and characterize the proteases resulting in ACE release under basal and antibody-induced conditions. The effect of mAbs on ACE shedding may provide a useful tool with which to dissect the cellular shedding apparatus.

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