Expression of Angiotensin-converting Enzyme Changes Major Histocompatiblity Complex Class I Peptide Presentation by Modifying C Termini of Peptide Precursors*

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We recently reported a mouse model called ACE 10/10 in which macrophages overexpress the carboxypeptidase angiotensin-converting enzyme (ACE). These mice have an enhanced inflammatory response to tumors that markedly inhibits tumor growth. Here, we show that ACE modifies the C termini of peptides for presentation by major histocompatibility complex (MHC) class I molecules. The peptide-processing activity of ACE applies to antigens from either the extracellular environment (cross-presentation) or antigens produced endogenously. Consistent with its role in MHC class I antigen processing, ACE localizes to the endoplasmic reticulum. ACE overexpression does not appear to change the overall supply of peptides available to MHC class I molecules. The immunization of wild type mice previously given ACE 10/10 macrophages enhances the efficiency of antigen-specific CD8+ T cell priming. These data reveal that ACE is a dynamic participant in fashioning the peptide repertoire for MHC class I molecules by modifying the C termini of peptide precursors. Manipulation of peptidase expression by antigen-presenting cells may ultimately prove a useful strategy to enhance the immune response.

Angiotensin-converting enzyme (ACE)3 is a zinc-dependent carboxy dipeptidase. ACE plays an important role in blood pressure regulation and electrolyte balance, mostly due to the conversion of angiotensin I to angiotensin II, from which its name is derived. However, ACE is somewhat promiscuous in that different peptides can be cleaved by the enzyme. For example, ACE efficiently degrades bradykinin (1). In vitro, ACE hydrolyzes enkephalins and neurotensins, as well as the β chain of insulin. ACE also shows activity against amidated peptides such as substance P and LH-RH (2). This wide substrate specificity is, in part, why ACE affects many different physiologic processes, including blood pressure, hematopoiesis, and fertility.

ACE is abundantly expressed in tissues such as vascular endothelium and renal tubular epithelial cells. Lesser amounts of ACE are made by many different tissue types (3). Antigen-presenting cells (APCs) make some ACE, with ACE expression induced during the differentiation of human monocytes into both macrophages (MØ) and dendritic cells (4, 5). Evidence has been presented that ACE may occasionally play an immunologic role. For example, ACE is important in the production of the immunodominant epitope of the human immunodeficiency virus 1 protein gp160 (6, 7). ACE has also been implicated in a variety of other inflammatory processes (8, 9).

Recently, we reported a mouse model called ACE 10/10 in which ACE is overexpressed in macrophage lineage cells (10). Surprisingly, these mice respond to the intradermal injection of the mouse melanoma cell line B16 with an enhanced inflammatory response that markedly limits tumor growth. Tumor challenge of ACE 10/10 mice with melanoma or lymphoma resulted in increased numbers of tumor-specific CD8+ T cells compared with wild type (WT) mice treated in an equivalent fashion. Enhanced tumor resistance was transferable to WT mice by bone marrow transplantation from ACE 10/10 mice. Although ACE 10/10 mice show several differences from WT mice, a central hypothesis for their increased immune response is that the increased production of the peptidase ACE by MØ may enhance the presentation of MHC class I-associated peptides to T cells.

Immune surveillance by CD8+ T cells requires the immune system to display thousands of peptides presented by MHC class I molecules (11). When a cell is infected with a microbe or has been transformed into a tumor, the peptide-MHC class I repertoire will now include non-self or new peptides derived from the infection or the neoplastic process. Further, bone marrow-derived APCs have a unique “cross-presentation” pathway by which APCs can internalize antigens from the extracellular environment and then present them as MHC class I-bound peptides (12, 13).

It is typically thought that the peptide generation pathway for MHC class I begins in the cytoplasm, where newly synthesized or phagocytosed polypeptides are fragmented by proteasomes. This cleaves proteins into peptides of roughly 5–20 amino acids (14). Peptide products are then shuttled into the endoplasmic reticulum (ER) via the transporter associated with antigen presentation (TAP). Processing of these peptides begins in the ER, is transported to the cis-Golgi, and is further processed in the TAP-positive endosomal pathway. The peptides are then transported to the plasma membrane and presented on MHC class I molecules for recognition by CD8+ T cells.
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processing. Once peptides arrive within the ER lumen, they are attacked by the ER aminopeptidase associated with antigen processing (ERAAP) and eventually destroyed if not protected by binding to a MHC class I molecule. It is generally thought that the C termini of peptides leaving the proteasomes already constitute the C terminus of the final MHC class I ligands and that, after binding to the MHC class I molecule, ERAAP removes N-terminal amino acid residues to yield the final peptide-MHC class I molecules that are eventually displayed on the cell surface (15, 16).

Here we report that ACE is able to modify the C termini of peptides for presentation by MHC class I molecules. This peptide-processing activity of ACE applies to antigens from either the extracellular environment (cross-presentation) or antigens produced endogenously. Our studies suggest that the modification of APCs by the overexpression of the carboxypeptidase ACE has the ability to manipulate the immune response and enhance the generation of CD8\(^+\) T cells. Manipulation of peptide expression by APCs may ultimately prove a useful strategy to enhance the immune response.

**EXPERIMENTAL PROCEDURES**

**Mice**—ACE 10 mice have been described (10). Briefly, by gene targeting, the promoter region of the somatic ACE gene was substituted with the c-fms promoter, resulting in ACE overexpression in macrophage lineage cells. ACE 10/10 mice were backcrossed ten times to the C57BL/6J (H-2b) background. C57BL/6 and FVB mice were purchased from Jackson Laboratory. OT-1 × RAG-1 knock-out mice, expressing Thy 1.1, were generously donated by Dr. Christian Larsen (Department of Surgery, Emory University School of Medicine). Mice were housed and bred in accordance with the guidelines of the Institutional Animal Care and Use Committee and the Department of Animal Resources at Emory University.

**Cells and Cell Lines**—Thioglycollate-elicited peritoneal exudate cells were collected via peritoneal lavage 4 days after a 2-ml injection of 3% thioglycollate broth intraperitoneally and were cultured in tissue culture-treated plates (1 × 10\(^6\)/ml) at 37 °C and 5% CO\(_2\) in 10% fetal calf serum RPMI 1640, 50 μM 2-ME, 0.5 mM sodium pyruvate, 10 mM HEPES buffer, 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine. For purification of thioglycollate-elicited peritoneal Mφ, peritoneal exudate cells were allowed to adhere for 2 h, after which nonadherent cells were washed off to achieve a >95% purity of Mφ. For flow cytometry assay, peritoneal exudate cells were treated and cultured in Costar low adherence culture plates. MACS\(^®\) with CD8\(^+\) T Cell Isolation kit (Miltenyi Biotec), which uses a negative selection strategy.

**Peptides and Reagents**—All peptides were synthesized by the Emory University Microchemical Core Facility. SAVKNYC-SKL and SAVKNYCSKL-CS were dissolved and used in solutions supplemented with 50 mM dithiothreitol. Some reagents were purchased: *Escherichia coli* 055:B5 LPS (Sigma), mouse IFN-γ (PeproTech), OVA (Worthington Biochemical), and Lisinopril (Sigma).

**Flow Cytometry**—The following antibodies were used for flow cytometry: anti-CD80, anti-CD40, anti-CD86, anti-CD69, anti-CD16/32, anti-H-2K\(^b\), and anti-H-2D\(^b\) (all from eBioscience), anti-F4/80 (AbD Serotec), and anti-I-A\(^b\) and anti-mouse IgG1 (both from BioLegends). A rabbit anti-serum that recognizes mouse ACE was previously described (18). The monoclonal antibody 25-D1.16 that recognizes K\(^b\)-SIINFEKL was kindly provided by Dr. Christian Larsen. After being stained with a standard protocol, cells were analyzed on a FACScalibur cytometer (BD Biosciences) with FlowJo software (TreeStar).

**Constructs and Transfections**—ACE, OVA, and GFP protein and minigene constructs were made using pcDNA3.1 (+) (Invitrogen). To make minigenes, two partially complementary oligonucleotides encoding the target peptide were designed. The 5′-end contained a BamHI site followed by the ribosome binding consensus sequence (5′-GCC GCC ACC-3′) and a methionine (ATG) for translation initiation. An XhoI site was designed at the 3′-end immediately following two translation stop codons (TAGTGA). Complementary oligonucleotides were allowed to anneal and ligated to BamHI- and XhoI-digested pcDNA3.1 (+). To make ER signal-leading peptide vectors, an ER signal sequence, AGGTACATGATTTTAG-GCTTGCTCGCCTTGGCGGACGTCTGACGGCT (23), was inserted between the initial ATG and the DNA backbone of the peptide antigens. L and L.K\(^b\) cells were transiently transfected with FuGENE\(^®\) HD (Roche Applied Science), and RMA-S cells were transfected with Nucleofection (AMAXA) (program X-001). Cells were harvested 24 h later for further assays.

**Antigen-presenting Assay**—In some experiments, antigen-pulsed MØ or transfected L.K\(^b\) cells were stained with 25-D1.16 antibody. In the experiments when OT-1 T cells or T hybridoma cells were used to measure antigen presentation, cells were fixed with 1% paraformaldehyde, washed, and then incubated with anti-OT-1 T or T hybridoma cells. OT-1 T cells were stained with anti-CD69 after 2 or 4 h of coincubation with OVA-pulsed Mφ. In other experiments, the supernatants of OT-1 T cells or T hybridoma cells were harvested after 16 h of coincubation with APCs, and IL-2 levels were assayed by enzyme-linked immunosorbent assay (eBioscience). For the in vivo experiment presented in Fig. 5, two groups of C57BL/6J recipients were transplanted intraperitoneally with 1.8 × 10\(^7\) thioglycollate-elicited peritoneal Mφ from either ACE 10/10 mice or WT littermates. Recipients also received intravenously 2 × 10\(^6\) OT-1 T cells labeled with CFSE (Molecular Probes). Five hours later, 400 μg of OVA was injected intraperitoneally into each recipient. Mice were harvested 40 h after this, and splenocytes were counted and analyzed by flow cytometry.
ACE ER Staining—To identify the distribution of ACE in subcellular organelles, resident ACE 10/10 peritoneal M\(^{\text{10}}\)/H\(^{\text{11083}}\) were purified with adherence and washes. ER-Tracker\(^{\text{TM}}\) Green was prepared according to the manufacturer's instructions (Molecular Probes). ACE 10/10 M\(^{\text{10}}\)/H\(^{\text{11083}}\) were washed with Hank's Balanced Salt Solution (HBSS/Ca/Mg) and incubated at 37 °C with 1/10262 M ER-Tracker Green for 30 min. Cells were then fixed in 4% paraformaldehyde for 2 min. 5-min phosphate-buffered saline washes were performed twice before a 10-min permeabilization consisting of 0.04% saponin in phosphate-buffered saline with 1% bovine serum albumin. Cells were then blocked with 1% bovine serum albumin and treated with rabbit anti-ACE serum. A secondary Alexa 546-labeled goat anti-rabbit antibody (Invitrogen) was used to stain ACE.

RESULTS

Effect of ACE Overexpression on the Presentation of Exogenous Antigens—To test whether ACE overexpression can change the processing of extracellular peptide antigens, we first examined the effects of ACE on the presentation of SIINFEKL, the dominant CD8\(^{+}\) T cell epitope of OVA presented by K\(^{b}\). The OVA system was chosen because ACE 10/10 mice, implanted with either the melanoma B16-OVA or the lymphoma EG.7, two OVA-expressing tumors, produced more OVA-specific CD8\(^{+}\) T cells than were found in WT mice. Peptide loading experiments were performed in the absence of serum, because serum contains active ACE. M\(^{\text{10}}\)/H\(^{\text{14135}}\) were loaded with SIINFEKL, and after 2 h the level of K\(^{b}\)-SIINFEKL complex on the cell surface was detected by flow cytometry using the monoclonal antibody 25-D1.16 (19) (Fig. 1A, left). An equivalent increase of signal intensity was observed with both ACE 10/10 and WT M\(^{\text{10}}\)/H\(^{\text{11083}}\). We then tested SIINFEKL-TE, a peptide containing SIINFEKL plus the next two carboxyl amino acids from the OVA sequence (Fig. 1A, right). M\(^{\text{10}}\)/H\(^{\text{11083}}\) were pulsed with this 10-mer peptide and again analyzed for surface expression of class I-associated SIINFEKL. ACE 10/10 M\(^{\text{10}}\)/H\(^{\text{11083}}\) showed more than 3-fold the efficacy of control cells. To verify that this was due to the overexpression of ACE by the ACE 10/10 cells, the experiment was also performed with increasing concentrations of a specific ACE inhibitor, lisinopril. At a lisinopril concentration of 100 nM, there was no difference between the two groups, although total enzyme inhibition was only achieved at 1 \(\mu\)M. Lisinopril is not toxic to cells, and 1 \(\mu\)M did not impair the presentation of exogenously added SIINFEKL. (Fig. 1A, left). These data indicate that ACE can process peptide present in an extracellular source for MHC class I presentation.

FIGURE 1. Presentation of extracellular peptides. Peritoneal M\(^{\text{10}}\) from WT (open bars) or ACE 10/10 (shaded bars) were collected, washed, and seeded into 96-well plates (2 \(\times\) 10\(^{4}\)/well). A, on the left, the cells were pulsed with 10 \(\mu\)M SIINFEKL for 2 h with or without the ACE inhibitor lisinopril (1 \(\mu\)M). On the right, M\(^{\text{10}}\) were treated with 10 \(\mu\)M SIINFEKL-TE in the presence of varying concentrations of lisinopril for 2 h. Surface K\(^{b}\)-SIINFEKL complex was detected for all groups with the 25-D1.16 antibody. Data are presented as mean fluorescence intensity (MFI). Bars are the mean ± S.E. B, M\(^{\text{10}}\)/H\(^{\text{11083}}\) were pulsed with the indicated concentration of peptides. After washing, cells were fixed with 1% paraformaldehyde. The presentation of SIINFEKL was detected by coincubating the M\(^{\text{10}}\) for an additional 16 h with 1 \(\times\) 10\(^{5}\) OT-1 hybridoma cells. Secreted IL-2 was measured by enzyme-linked immunosorbent assay (mean ± S.E.). C, an experiment equivalent to that in B but now using the peptide SAVKNYCSKL-CS and the T hybridoma HLT359. For all data in this figure, *, \(p < 0.05\); **, \(p < 0.01\); ***, \(p < 0.005\); \(n = 6\).
We also tested the relationship of peptide length to ACE-dependent activation of T cells by using 11- and 12-mer peptides, which have 3 or 4 amino acids beyond the C-terminal of SIINFEKL. After a 2-h incubation with peptides, MØ were fixed with paraformaldehyde and the ability to present SIINFEKL was measured by IL-2 secretion from an OT-1 T cell hybridoma. With both the 11- and 12-mer peptides, ACE 10/10 MØ were more efficient in trimming than WT MØ (Fig. 1B). For example, ACE 10/10 MØ stimulated 2.2-fold more IL-2 production by T cells when 0.5 μM of the 12-mer peptide was used.

To further study whether ACE can modify peptides for MHC class I presentation, SAVKNYCSKL, the dominant Db-represented antigen, and the C-terminal dipeptide extended peptide SAVKNYCSKL-CS were pulsed onto MØ. This peptide and the C-terminal dipeptide were transiently cotransfected into L.Kb cells. Control cells were cotransfected with OVA, empty vector, and GFP (which allowed transfected cells to be identified). Also, GFP expression in cells cotransfected with GFP and OVA (data not shown). Finally, we measured surface MHC class I expression in ACE-overexpressing cells and found no difference from control cells (see Fig. 6C). Therefore, ACE overexpression is not toxic, does not change the MHC class I maturation, and can significantly increase OVA presentation without interfering with plasmid transfection and expression of transfected genes.

To further investigate the possible mechanism underlying the increased presentation of OVA, we studied the effects of overexpression of ACE on the presentation of the 10-mer SIINFEKL-TE and the 11-mer SIINFEKL-TEW expressed in the cytoplasm as synthetic minigenes. In this experiment, the peptides are direct translation products rather than products of proteasome-mediated degradation of precursor peptides. Constructs are synthesized with an initiating methionine that is predicted to be removed by methionyl aminopeptidase (23). Even without ACE cotransfection, L.Kb cells had the ability to produce some SIINFEKL from these C-terminal-extended precursors (Fig. 3B). However, a marked increase was noted with ACE co-expression. For example, the mean fluorescence intensity of cells transfected with the SIINFEKL-TE peptide and ACE was 3.1-fold that of cells lacking ACE after analysis with 25-D1.16 antibody (Fig. 3A). ACE overexpression significantly increased the production of SIINFEKL epitope in a dose-dependent manner. In addition, this ACE-mediated increase could be completely eliminated by treatment with lisinopril. Trypan blue exclusion analysis showed that there was no change in viability in ACE-overexpressing cells (data not shown). Also, GFP expression in cells cotransfected with GFP and ACE was similar to those cotransfected with GFP and OVA (data not shown). Finally, we measured surface MHC class I expression in ACE-overexpressing cells and found no difference from control cells (see Fig. 6C). Therefore, ACE overexpression is not toxic, does not change the MHC class I maturation, and can significantly increase OVA presentation without interfering with plasmid transfection and expression of transfected genes.

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If ACE activity can modify the C termini of epitope precursors, we might also predict some epitopes themselves would be ACE substrates. To examine such an epitope, we studied RRLGRTLLL, which is derived from the polyoma virus middle T antigen (MT389–397) and is presented by Dk (24). The effect of ACE overexpression on the presentation of this epitope from full-length MT protein was studied first. L cells, which constitutively express Dk, were cotransfected by either MT with ACE or MT with control GFP vector. IL-2 secretion by the MT389–397-specific T hybridoma H8-1.18 was then quantified (Fig. 3C, left). ACE expression had the effect of destroying rather than facilitating the production of RRLGRTLLL. Using a different experimental approach, we found that L cells transfected with a RRLGRTLLL minigene alone stimulated H8-1.18 cells to a greater extent than L cells receiving minigene plus ACE (Fig. 3C, right). Although we did not directly determine the amino acid sequence of presented peptides, in aggregate the data in Fig. 3 suggest that ACE expression leads to C-terminal trimming of the RRLGRTLLL sequence.

Catalytically Active ACE Is Located in the ER—The discovery that ACE can be involved in both cross-presentation of OVA and presentation of endogenous antigens in ACE 10/10 M/H11083 prompted the question of how ACE can reach antigens inside cells. Normally, ACE is exported to the cell membrane where it is bound by its C-terminal hydrophobic sequence (40). To elucidate the intracellular distribution of ACE, we co-stained ACE 10/10 M/H11083 with ER-Tracker Green, a small molecule that localizes to the ER, and a polyclonal ACE antibody. When we stained live cells without fixation or permeabilization, ACE was identified on the cell membrane, whereas the ER-Tracker Green stained the ER (×20 objective). For the bottom row, cells were first stained with ER-Tracker Green and then fixed, permeabilized, and stained with anti-ACE. Intracellular ACE is seen to co-localize with the ER (×60 objective). B, RMA-S cells were transfected with minigenes encoding either SIINFEKL or SIINFEKL-TE and an N-terminal signal sequence. Some groups were also cotransfected with the ACE gene. After 24 h, the percentage of cells with positive 25-D1.16 antibody staining was determined. The data from individual samples are presented, as well as the group mean ± S.E. p = 0.01 when comparing the SIINFEKL-TE groups with or without ACE cotransfection.

ACE Trims C Termini of Class I Peptide Precursors—The discovery that ACE can be involved in both cross-presentation of OVA and presentation of endogenous antigens in ACE 10/10 M/H11083 prompted the question of how ACE can reach antigens inside cells. Normally, ACE is exported to the cell membrane where it is bound by its C-terminal hydrophobic sequence (40). To elucidate the intracellular distribution of ACE, we co-stained ACE 10/10 M/H11083 with ER-Tracker Green and rabbit anti-ACE serum followed by Alexa 546 (red)-labeled goat anti-rabbit IgG. Top row, without fixation or permeabilization, anti-ACE stained the cell membrane while the ER-Tracker Green stained the ER (×20 objective). For the bottom row, cells were first stained with ER-Tracker Green and then fixed, permeabilized, and stained with anti-ACE. Intracellular ACE is seen to co-localize with the ER (×60 objective). B, RMA-S cells were transfected with minigenes encoding either SIINFEKL or SIINFEKL-TE and an N-terminal signal sequence. Some groups were also cotransfected with the ACE gene. After 24 h, the percentage of cells with positive 25-D1.16 antibody staining was determined. The data from individual samples are presented, as well as the group mean ± S.E. p = 0.01 when comparing the SIINFEKL-TE groups with or without ACE cotransfection.
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FIGURE 5. ACE 10/10 MØ present OVA more efficiently in vivo. WT C57BL/6 recipients received 1.8 × 10⁷ MØ intraperitoneally from ACE 10/10 or WT mice and were injected intravenously with 2 × 10⁷ CFSE-labeled Thy1.1 OT-1 T cells. A, 5 h after cell transfer, 400 μg of OVA was injected intraperitoneally into each recipient. 45 h after cell transfer, mice were sacrificed and splenocytes were analyzed by FACS. Plots are gated on Thy1.1 positive cells. Results are representative of six mice in each group. B, similar to A panels but MØ and OT-1 T cells were transferred without OVA injection. C, SIINFEKL peptide-pulsed MØ and OT-1 T cells were transferred without OVA administration. The results are representative of five mice in each group.

tom row). Therefore, abundant ACE resides in the ER where it may be available to modify intracellular peptides, including peptides for the formation of mature MHC class I.

To further characterize ACE within the ER, we examined the effect of ACE transfection on the presentation of an antigenic peptide delivered directly into the ER, avoiding exposure to cytosolic enzymes. This was done in the transporter associated with antigen processing-defective cell line RMA-S (H-2b) to avoid the possibility that ER peptides would leak into the cytosol and then be transported back to the ER. Either SIINFEKL or SIINFEKL-TE minigenes with an added N-terminal signal sequence were expressed in RMA-S cells. The signal sequence causes peptides to be transported into the ER immediately after translation by ribosomes but is itself degraded shortly thereafter (25). RMA-S cells were transfected and, 24 h later, studied by FACS using the 25-D1.16 antibody. As shown in Fig. 4B, RMA-S cells rapidly and efficiently presented SIINFEKL, but not SIINFEKL-TE. However, cotransfection with ACE allowed the RMA-S cells to process the C-terminal extension and present SIINFEKL-TE with the same efficiency as SIINFEKL. Thus, these experiments with ER-directed peptides, as well as the immunochromatographic data presented above, show ACE is present in the ER lumen and can modify the C termini of MHC class I precursor peptides.

ACE Promotes Antigen Presentation to CD8⁺ T Cells in Vivo—The tumor models studied in ACE 10/10 mice suggested that ACE overexpression in MØ could influence and even augment antigen presentation to CD8⁺ T cells in vivo. Here, we used an adoptive transfer strategy to investigate this hypothesis. C57BL/6 recipients received 1.8 × 10⁷ MØ intraperitoneally either from WT or ACE 10/10 donor mice. At the same time, the recipient mice were also transfused intravenously with 2 × 10⁶ Thy1.1 OT-1 T cells that were previously labeled with CFSE. Five hours later OVA was injected intraperitoneally, and 45 h later mice were sacrificed and the division pattern and cell number of splenic OT-1 T cells were evaluated (Fig. 5A). Although the total number of splenocytes from both groups was approximately the same, the number of OT-1 T cells from the group of mice that had received ACE 10/10 MØ averaged 4.26 ± 0.49 × 10⁶ cells per spleen versus 2.41 ± 0.41 × 10⁶ cells in the spleens of mice that had received control MØ (p < 0.02, n = 6). Further, the pattern of CFSE dye dilution indicated a clear difference in the number of proliferative cells; OT-1 T cells isolated from mice implanted with ACE 10/10 MØ showed increased proliferation as compared with the cells collected from mice implanted with WT MØ.

To exclude the possibility that transferred ACE 10/10 MØ nonspecifically activated the recipients’ immune system, recipients received MØ and OT-1 T cells but no OVA. Now there was no evidence of OT-1 proliferation (Fig. 5B). Further, to ensure that the differences seen in Fig. 5A were not due to a nonspecific higher efficacy of presentation by ACE 10/10 MØ (other than due to the overexpression of ACE), MØ were pulsed with SIINFEKL peptide in vitro, washed, and then injected into recipients. In this control experiment, there was no further immunization with OVA. The division patterns of OT-1 cells were very similar between the two recipient groups (Fig. 5C), and there was no significant difference in the total number of OT-1 cells in the spleens (2.24 ± 0.28 × 10⁶ of ACE 10/10 recipients versus 2.35 ± 0.27 × 10⁶ of WT ones, n = 5).

Effect of ACE Overexpression on the Levels of Surface Costimulatory and MHC Molecules—The presentation of peptides bound to MHC class I can be influenced by a variety of factors apart from the hydrolysis of peptides. To exclude the possibility that there is a difference in maturation state between ACE 10/10 and WT MØ, we characterized peritoneal MØ for surface expression of costimulatory and MHC class II molecules. For both non-stimulated and LPS-induced ACE 10/10 MØ, the expression of CD40, CD80, CD86, and I-A<sup>B</sup> was not different from that of WT MØ (Fig. 6A).

Newly synthesized MHC class I complexes are unstable and are retained in the ER until they bind peptide, after which the stable complexes are transported to the cell surface (26). The generation of stable MHC class I molecules and their expression on the cell surface depends on peptide supply, so changes in peptide supply can be assessed by differences in surface MHC class I levels. We evaluated the surface expression of MHC class I on cells overexpressing ACE. First we measured the levels of Kb and D<sup>b</sup> on ACE 10/10 MØ and found that the constitutive level of these antigens was similar to levels on WT MØ (Fig. 6B). As an additional control, we measured MHC class I levels of MØ treated with IFN-γ (27) or LPS (28), because these agents can enhance overall antigen presentation. Even under these conditions, surface levels of K<sup>b</sup> and D<sup>b</sup> were similar to WT. Finally, LK<sup>b</sup> cells were transfected with either ACE or OVA. Both groups showed no significant difference in surface K<sup>b</sup> (Fig. 6C). The observation of normal levels of surface MHC class I suggests that overexpressing ACE has little effect on the total number of self-antigen-derived MHC class I peptides in the intracellular pool.

Roles of ACE in Wild Type Macrophages—Our studies have focused on ACE 10/10 MØ that overexpress ACE. However, certain of our data raise the question of the role of ACE in WT
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For example, in Fig. 1A, right, the ability of WT MØ to present Kb-SIINFEKL after being pulsed with SIINFEKL-TE was inhibited by increasing doses of lisinopril. To understand the role of ACE in WT MØ, we used FACS to measure the expression of ACE in WT, ACE 10/10, and MØ derived from a line of ACE knock-out mice termed ACE 4/4 (29). These data show that WT MØ express only a small amount of ACE, at least under the conditions used to prepare these cells (Fig. 7A). ACE 4/4 MØ completely lack ACE due to genetic manipulation; they were used in two experimental protocols to evaluate peptide processing. In the first protocol, MØ were loaded with 10 μM SIINFEKL-TE, and after 2 h the level of Kb-SIINFEKL complex was evaluated using the 25-D1.16 antibody. ACE 4/4 cells showed virtually no ability to process and present SIINFEKL-TE in this time period (data not shown) compared with the 6% efficiency of WT MØ (Fig. 1A, right), which further proves that this extracellular C-terminal-extended peptide could only be trimmed by ACE in MØ. In another protocol, we loaded ACE 4/4, WT, and ACE 10/10 MØ with OVA. Here, presentation of Kb-SIINFEKL was measured by the IL-2 production of OT-1 hybridoma cells. As shown in Fig. 7B, ACE 4/4 cells were almost equivalent to WT in their capacity to process OVA. In contrast, ACE 10/10 cells induced approximately twice the levels of IL-2. These experiments suggest that ACE probably plays a minimal role in antigen processing under basal conditions, because only small amounts of this enzyme are made by WT MØ. However, when levels of ACE are increased, as in ACE 10/10 cells, this peptidase now has the capacity to alter the presentation and processing of peptides.

DISCUSSION

The studies presented here were undertaken after observing that ACE 10/10 mice respond to the intradermal implantation of melanoma with an enhanced inflammatory response, resulting in a marked reduction of tumor growth. This was dependent on CD8+ T cells. Further analysis with T cell receptor tetramer reagents consistently found enhanced numbers of CD8+ T cells directed against Trp-2, an intrinsic tumor marker, and OVA, an ectopic marker (10). ACE 10/10 mice are unique in that ACE expression is controlled by the c-fms promoter. This results in a high level of ACE expression by monocytes, MØ, for example, in Fig. 1A, right, the ability of WT MØ to present Kb-SIINFEKL after being pulsed with SIINFEKL-TE was inhibited by increasing doses of lisinopril. To understand the role of ACE in WT MØ, we used FACS to measure the expression of ACE in WT, ACE 10/10, and MØ derived from a line of ACE knock-out mice termed ACE 4/4 (29). These data show that WT MØ express only a small amount of ACE, at least under the conditions used to prepare these cells (Fig. 7A). ACE 4/4 MØ completely lack ACE due to genetic manipulation; they were used in two experimental protocols to evaluate peptide processing. In the first protocol, MØ were loaded with 10 μM SIINFEKL-TE, and after 2 h the level of Kb-SIINFEKL complex was evaluated using the 25-D1.16 antibody. ACE 4/4 cells showed virtually no ability to process and present SIINFEKL-TE in this time period (data not shown) compared with the 6% efficiency of WT MØ (Fig. 1A, right), which further proves that this extracellular C-terminal-extended peptide could only be trimmed by ACE in MØ. In another protocol, we loaded ACE 4/4, WT, and ACE 10/10 MØ with OVA. Here, presentation of Kb-SIINFEKL was measured by the IL-2 production of OT-1 hybridoma cells. As shown in Fig. 7B, ACE 4/4 cells were almost equivalent to WT in their capacity to process OVA. In contrast, ACE 10/10 cells induced approximately twice the levels of IL-2. These experiments suggest that ACE probably plays a minimal role in antigen processing under basal conditions, because only small amounts of this enzyme are made by WT MØ. However, when levels of ACE are increased, as in ACE 10/10 cells, this peptidase now has the capacity to alter the presentation and processing of peptides.

DISCUSSION

The studies presented here were undertaken after observing that ACE 10/10 mice respond to the intradermal implantation of melanoma with an enhanced inflammatory response, resulting in a marked reduction of tumor growth. This was dependent on CD8+ T cells. Further analysis with T cell receptor tetramer reagents consistently found enhanced numbers of CD8+ T cells directed against Trp-2, an intrinsic tumor marker, and OVA, an ectopic marker (10). ACE 10/10 mice are unique in that ACE expression is controlled by the c-fms promoter. This results in a high level of ACE expression by monocytes, MØ.
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and other macrophage lineage cells such as Kupfer cells. Although several different mechanisms may contribute to the enhanced immune response present in ACE 10/10 mice, the overexpression of the peptidase ACE by monocytes and MØ raised the question of whether this enzyme played a role in peptide processing and presentation.

In APCs, it is well established that peptides presented on MHC class I molecules are byproducts of the continual degradation of cell proteins by the proteasome pathway. Proteasomes can directly generate mature epitopes, but isolated proteasomes appear to preferentially generate terminal-extended precursors (22). A number of studies with model peptides have shown that cellular aminopeptidases are able to rapidly process N-extended peptides to mature epitopes, and several aminopeptidases performing this function in vivo have been identified (15, 16, 30, 31). However, it is unclear to what extent carboxyl peptidases participate in the trimming of peptides necessary to generate MHC class I epitopes or whether proteasomes universally make the exact C termini of the final epitopes.

In this report, we have presented several different experimental approaches showing that the overexpression of ACE can result in changes in the ability of cells to process and present MHC class I peptides. A variety of antigen sources were systemically evaluated, from peptide precursors to proteins and from extracellular antigens to endogenous ones. We also examined epitopes presented on different MHC class I molecules, including OVA257–264 (an 8-mer peptide presented by Kb), LT359–368 (a 10-mer peptide presented by Db), and MT389–397 (a 9-mer peptide presented by Dd). The sensitivity of the antigen processing to the ACE inhibitor lisinopril provides strong evidence that it is ACE catalysis mediating antigen processing. These studies culminated with the in vivo data (Fig. 5) showing enhanced generation of antigen-specific CD8+ T cells in immunized mice implanted with ACE 10/10 MØ, as assessed using the OVA/OT-1 system. Our work echoes the study of Eisenlohr et al. (32), who showed that ACE-transfected fibroblasts were more efficient than WT cells in trimming the carboxyl extensions of influenza-derived peptides into MHC class I-presented peptides recognized by CD8+ cytotoxic lymphocytes. As indicated, one view about the production of MHC class I peptides is that the C termini of peptides leaving proteasomes already constitute the final C termini of MHC I ligands (33–35). Actually, when Cascio et al. (22) purified 26 S proteasomes and examined the peptides made from OVA protein in vitro, they found that the total 1- and 2-mer C-terminal-extended SIINFEKL peptides were generated at a rate comparable with the total 1- and 2-mer N-terminal-extended peptides (1.2 versus 1.4%). The percentage of C-terminal-extended peptides was even higher when the products of core 20 S proteasomes were examined. Therefore, in terms of OVA protein, there could be abundant C-terminal-extended SIINFEKL peptides produced by proteasomes. If ACE is overexpressed, ACE may change the spectrum of C termini originally made by proteasomes and consequently may change the peptide repertoire for MHC I molecules as well.

The importance of our studies is that the overexpression of ACE, and perhaps other carboxypeptidases, changes the immune response of an animal, at least in part by changing antigen processing. ACE, like any enzyme, has preferences in substrate selection (36). Whether the addition of carboxypeptidase activity to antigen-processing cells in vivo will always enhance an immune response is not known. However, this represents a new approach to the manipulation of the immune system and, at least as far as is indicated by the ACE 10/10 model, a manipulation that has the potential to significantly enhance the immune response to tumors such as melanoma and lymphoma.

Although ACE undoubtedly functions in the Mφ of ACE 10/10 mice to trim peptides, the situation in WT cells is less certain. WT MØ make little ACE, but these cells do appear different from MØ lacking all ACE. In particular, some processing of SIINFEKL-TE does occur in WT MØ, compared with virtually none in MØ from ACE KO animals, and this can be inhibited by lisinopril. However, the limited quantities of ACE made under natural circumstances, and the lack of any suggested antigen-processing defect in human patients on ACE inhibitors, implies a limited role of ACE in most circumstances. Still, it is worth noting that ACE is up-regulated when human monocytes are stimulated by IFN-γ (37). Also, in granulomatous diseases, such as sarcoid or leprosy, there is enhanced ACE production by macrophage-like cells (38, 39). Whether these are associated with an enhanced immune response is not known.

In summary, our data show that the overexpression of ACE in APCs is associated with changes in the processing and presentation of antigens associated with MHC class I molecules. This is due to the carboxypeptidase activity of ACE. Depending on the peptide epitope, ACE expression can have a positive or negative contribution to the generation of individual class I epitopes. In ACE 10/10 mice, overexpression of ACE by monocytes and MØ is associated with enhanced resistance to the growth of melanoma. Whether manipulation of peptidase expression by APCs can be used as a form of immune enhancement for tumors or chronic infections remains to be investigated.

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