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Carbonic Anhydrases in Chick Extra-embryonic Structures: A Role for CA in Bicarbonate Reabsorption Through the Chorioallantoic Membrane

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The villus cavity cells, a specific cell type of the chick chorioallantoic membrane, express both cytosolic carbonic anhydrase in their cytoplasm and HCO_3^-/Cl^- anion exchangers at their basolateral membranes. By immunohistochemical analysis, we show here that villus cavity cells specifically react with antibodies directed against the membrane-associated form of carbonic anhydrase, CAIV. Staining is restricted to the apical cell membranes, characteristically invaginated toward the shell membrane, as well as to endothelia of blood vessels present in the mesodermal layer. The occurrence of a membrane-associated CA form at the apical pole of villus cavity cells, when definitively confirmed, would be fairly consistent with the role proposed for these cells in bicarbonate reabsorption from the eggshell so to prevent metabolic acidosis in the embryo during development.

Keywords: Carbonic anhydrase; Chorioallantoic membrane; Villus cavity cells; Bicarbonate reabsorption; Chick development

INTRODUCTION

The chick chorioallantoic membrane (CAM) is an extra-embryonic structure which forms, during development, by fusion of chorion and allantois. This results in a three-layered structure consisting of the chorionic epithelium in contact with the shell membrane, the mesodermal middle layer, and the allantoic epithelium lining the allantoic cavity.[1,2] The chick CAM serves multiple functions in the embryo morphogenesis: it is the site of exchange of respiratory gases, Ca^{2+} transport from the eggshell, and ion and H_2O reabsorption from the allantoic fluid.[3,4] The cytosolic form of carbonic anhydrase is present in both the CAM epithelial layers. It has been demonstrated that the enzyme expression is developmentally regulated and restricted to specific cell types, the villus cavity (VC) cells in the chorionic epithelium, and the mitochondria-rich cells in the allantoic epithelium.[2] The finding that VC cells express also AE1 anion exchangers at their basolateral membranes[5] supports a role for these cells in bicarbonate reabsorption that is required in the embryo for maintaining acid-base balance during development.[6–8] In view to further elucidate the molecular components or mechanisms by which transport of HCO_3^- ions, originated from the eggshell, can be accomplished through the chorionic VC cells, antibodies specific for the carbonic anhydrase isoenzyme CAIV have been used to explore, by an immunohistochemical analysis, the expression of this membrane-associated CA form in the chick CAM.

MATERIALS AND METHODS

Antibodies and Reagents

Antibodies employed in this study comprise the sheep polyclonal antibody directed against the active site peptide (aminoacids 125–138) of human CAIV and the rabbit polyclonal antibody directed against an N-terminal peptide of human CAIV.[9] These antibodies were a generous gift of Dr. Nicholas D. Carter (Med.Genetics Unit, St.George's Hospital Medical School, London, UK).

The reagents, if not otherwise indicated, were from Vector Laboratories (Burlingame, CA, USA).

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Immunohistochemistry

Embryos of Cobb chickens at day 16 of incubation were used. The CAM tissues, removed together with the shell membranes, were fixed in Bouin's solution for 3 h at room temperature. Samples were dehydrated, cleared in xylene and embedded in paraffin wax at 56–58°C. Rehydrated sections (5 μm thick) were first processed for inactivation of endogenous peroxidase (0.3% H_2O_2 in methanol for 30 min) and blocking of endogenous avidin-binding activity (avidin–biotin blocking kit). Non-specific interactions were prevented by incubation for 20 min in either normal rabbit serum or normal goat serum, diluted 1:5 with 1% bovine serum albumin (BSA, Sigma Chemical Co., St.Louis, MO, USA) in 0.05 M phosphate-buffered saline (PBS), pH 7.6. Sections were then incubated overnight with either the sheep antibodies directed against the human CAIV active site or the rabbit antibodies against the human CAIV N-terminal peptide. Both antibodies were diluted 1:300 with PBS containing 1% BSA. After washing in PBS (3 × 5 min), sections were incubated with biotinylated rabbit anti-sheep IgG or biotinylated goat anti-rabbit IgG (1:400 in PBS) for 45 min, rinsed in PBS and treated with the avidin-biotinylated peroxidase complex (ABC Elite) with 3,3′-diaminobenzidine (DBA kit) as a chromogen substrate. Finally, sections were dehydrated and mounted with Eukitt (Kindler GmbH & Co., Freiburg, Germany). In control sections, the primary antibodies were replaced with PBS containing 1% BSA.

RESULTS

Both antisera directed against the membrane-associated CAIV produced similar staining patterns in the chick CAM sections which showed punctuate sites of immunoreactivity, scattered at the upper surface of the CAM toward the shell membrane. Based on the anatomical structure of the chick CAM (Figure 1), it can be stated that the immunoreactive sites are located in the chorionic epithelium while they are absent in the allantoic epithelium (Figure 2A). In the middle layer, endothelial cells of blood vessels are stained. Within the chorionic epithelium, staining is restricted to some cells, interspersed among unreactive cells, and it is located at the apical cell membranes which invaginate in depressions between adjacent vessels of the intrachorionic blood sinus (Figure 2B). This feature of the apical cell membrane is specific to a distinct cell type, the villus cavity (VC) cells which alternate with capillary covering (CC) cells within the chorionic epithelium (Figure 1).

No staining was detected in control sections incubated with PBS instead of primary antibody (Figure 3).

FIGURE 1 A schematic representation of the chick CAM. The morphology of the distinct cell types of the chorionic epithelium is indicated. VC, villus cavity cells; CC, capillary covering cells. 1, eggshell; 2, shell membrane; 3, chorionic epithelium; 4, mesodermal layer; 5, allantoic epithelium; 6, allantoic cavity.

DISCUSSION

This immunohistochemical study indicates that antibodies against the membrane-associated CAIV isoform react specifically with the chorionic epithelium of the chick CAM. Distribution patterns show that immunostaining is located at the apical cell membrane of cells which, on the basis of their structural features, can be identified as VC cells. In this cell type, specific expression of the cytosolic CAII[2] was previously related to production of H^+ ions which would be released apically via a vacuolar-type H^+-ATPase present at the apical pole of the VC cells.[8] Proton secretion results in regional acidification thereby causing solubilization of the eggshell mineral calcite. Hence, Ca^{2+} ions become available to be reabsorbed into the embryo through the chorionic epithelium by mechanisms not yet fully elucidated.[1,4] Also bicarbonate ions are mobilized, thus providing the main source of extra-embryonic bicarbonate supply which is required to prevent metabolic acidosis in the embryo during development.[7,8] The recent demonstration that AE1 anion exchangers, which mediate the exchange of Cl^- for HCO_3^- ions in erythroid cells and in type A-intercalated cells of mammalian and avian kidney,[11–15] are specifically expressed at the VC cell basolateral membranes fairly agrees with a process of bicarbonate reabsorption from the eggshell, and candidates the VC cells as the specialized sites of this transport.[10] In such a context, mechanisms promoting HCO_3^- influx from the eggshell into the VC cells might be requested, such as apical Na^+/HCO_3^- cotransporters as already established in several tissues.[16–20] The present finding that molecular components, present at the apical cell membrane of VC cells, are recognized by antisera specific for human CAIV suggests an alternative, although not conclusive, interpretation. A membrane-associated form of carbonic anhydrase, which shares antigenic

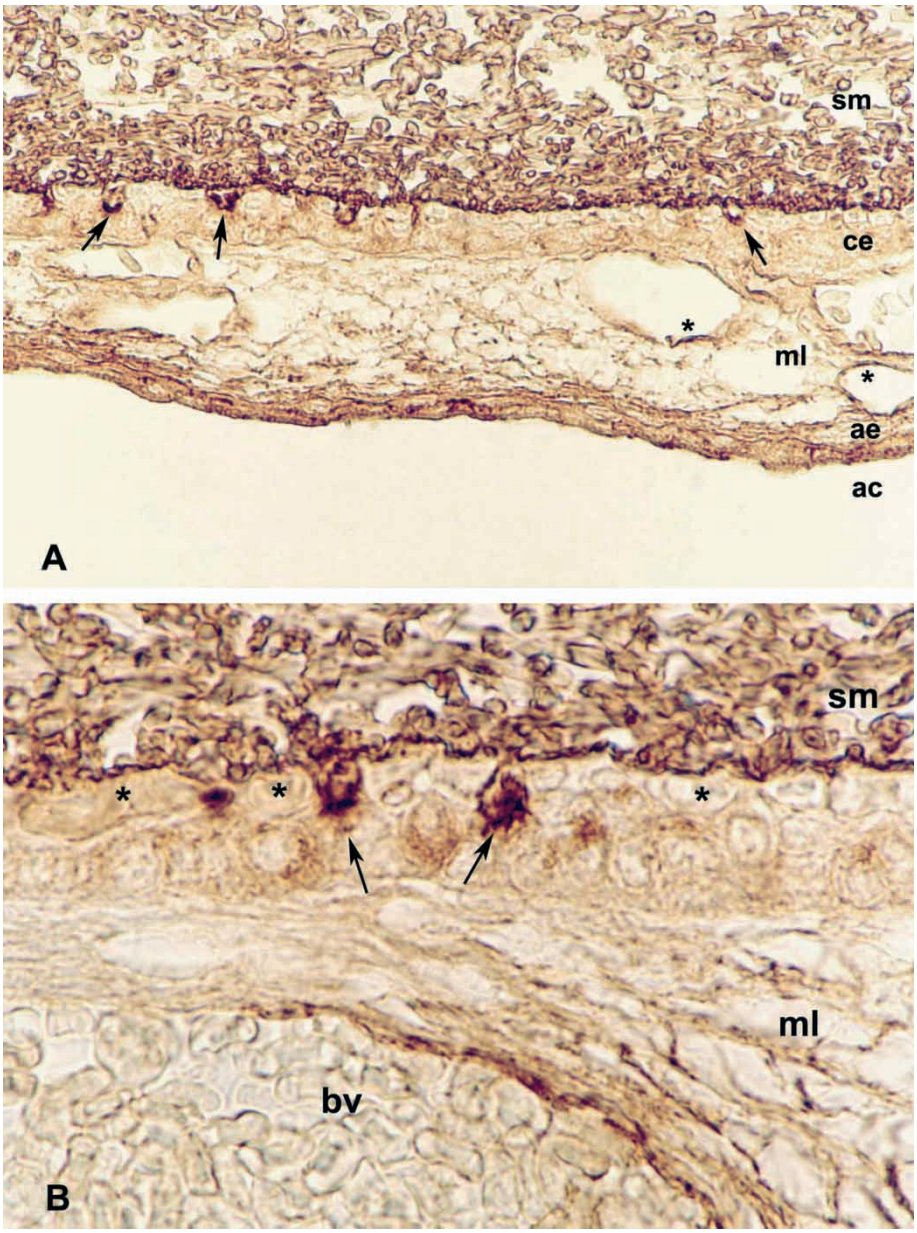

FIGURE 2 Chick CAM of 16 day-old embryo after incubation with anti CAIV active site.(A) Immunoreactive sites (arrows) can be detected, scattered within the chorionic epithelium (ce) toward the shell membrane (sm). The mesodermal layer (ml) and allantoic epithelium (ae) are unstained. Endothelia of mesodermal vessels show some staining (asterisks). Allantoic cavity (ac). (× 400) (B) High magnification of the chorionic epithelium. In the reactive cells, the staining is restricted to the apical membranes (arrows) and does not extend to the cytoplasm and basolateral membranes. Asterisks, intrachorionic blood sinus; bv, mesodermal blood vessel (× 1100).

properties with human CAIV, may exist at the VC cell apical pole. At this site, analogously with CAIV isoenzyme, it might promote the conversion of HCO_3^- ions, originated from the eggshell dissolution, to CO_2 which can diffuse promptly across the apical membrane into the cells. In the VC cell cytoplasm, CO_2 would be converted to HCO_3^- ions by the cytosolic CAII and then would leave the basolateral pole via AE1 anion exchangers.

The occurrence in avian tissues of a membrane-associated CA form, with distribution and potential roles similar to those of mammalian CAIV, was previously indicated by indirect evidence. Comparative analysis of results obtained by histochemistry and immunohistochemistry in quail kidney and lower intestine[21–23] pointed out differences in distribution of reactive sites thus raising the question as to the existence of additional forms of the enzyme, antigenically distinct from the known cytosolic forms. This hypothesis had been initially proposed on the basis of the dose-dependent effects that the CA inhibitor, acetazolamide, proved to have on development of the histochemical reaction.[24]

Additional investigations are in progress to confirm and substantiate the preliminary results of

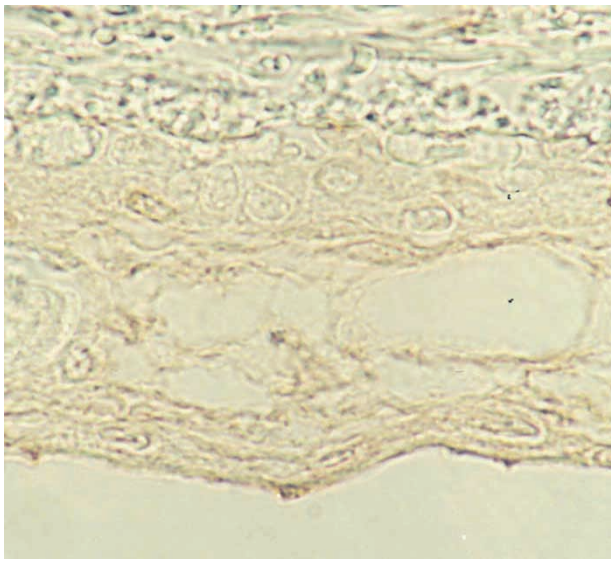

FIGURE 3 Chick CAM. No staining is present when sections are incubated with PBS instead of the primary antibody (× 850).

our study as well as to support the functional model for VC cells, as proposed here.

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