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Specialised cell types in the chorioallantoic membrane express carbonic anhydrase during chick embryogenesis

M. GABRIELLA GABRIELLI[1], GIOVANNI MATERAZZI[1], JOHN V. COX[2] AND GIOVANNA MENGHI[1]

[1]*Department of Comparative Morphological and Biochemical Sciences, University of Camerino, Camerino, Italy, and* [2]*Department of Microbiology and Immunology, University of Tennessee Health Science Center, Memphis, Tennessee, USA*

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ABSTRACT

The expression of carbonic anhydrase in the chorioallantoic membrane (CAM) of the chick embryo was investigated by means of the histochemical localisation of the enzyme catalytic sites and the immunohistochemical identification of its isoenzymatic forms. The results show that carbonic anhydrase is developmentally expressed in a subset of cells both in the ectodermal and the endodermal epithelium. The distribution patterns from both methodological approaches indicated that carbonic anhydrase is a marker of the villus cavity cells and the mitochondria-rich cells in the ectodermal and the endodermal epithelium, respectively. Such a cell-specific pattern of the enzyme expression provides a further contribution to characterising the heterogeneous cell population of the chick CAM and supports specific functional involvement for the distinct cell types in CAM-mediated processes, such as calcium transport, maintenance of acid-base balance and water and electrolyte reabsorption, during chick embryogenesis.

Key words: Avian development; extra-embryonic structures.

INTRODUCTION

In examining the contribution of extra-embryonic tissues to chick morphogenesis, extensive studies have focused on the role of the chorioallantoic membrane (CAM). The chick CAM is formed as the result of the progressive fusion of the chorionic and allantoic membranes so that it consists of 3 distinct cellular layers: an ectodermal epithelium adjacent to the eggshell, a mesodermal middle layer belonging to both chorion and allantois, and an endodermal epithelium lining the allantoic cavity (Leeson & Leeson, 1963; Packard & Packard, 1984). By incubation d 10–11, the CAM surrounds the embryo and adheres to the acellular inner shell membrane (Freeman & Vince, 1974). Concurrent with CAM differentiation, an extensive blood sinus differentiates towards the shell membrane, eventually covering the entire expanse of the chorion (Narbaitz, 1977; Ausprunk, 1986). By this vascular arrangement, which probably makes the blood circulation rather slow, and by its position immediately subjacent to the porous shell, embryonic gaseous exchange with the environment is facilitated. As morphogenesis progresses, the CAM undergoes morphological changes and expresses enzymatic activities that testify to an active transport of calcium from the shell to the embryo, thereby contributing to skeletal mineralisation (Terepka et al. 1969; Tuan et al. 1986). At the same time, the CAM is engaged both in maintaining acid-base homeostasis (Dawes & Simkiss, 1969, 1971; Narbaitz et al. 1995) and in regulating electrolyte and water reabsorption in the embryo (Stewart & Terepka, 1969; Simkiss, 1980). Several studies have attempted to relate these different functions to the structural features of the ectodermal and endodermal epithelia as well as to define the mechanisms by which they are accomplished. Of the functional markers investigated, the enzyme carbonic anhydrase (CA), based on its catalytic activity, seems likely to represent a multivalent factor acting in the different aspects of the CAM physiology. Indeed, carbonic anhydrase, by catalysing the interconversion of carbon dioxide and bicarbonate, participates in a variety of physiological

Correspondence to Dr Maria Gabriella Gabrielli, Department of Comparative Morphological and Biochemical Sciences, University of Camerino, Via Gentile III da Varano, I-62032 Camerino (MC), Italy. Fax: +(0)737-402708; e-mail: gabriell@camserv.unicam.it

processes that involve pH regulation, ion transport and water and electrolyte balance (Maren, 1967; Carter, 1972; Sly & Hu, 1995; Parkilla & Parkilla, 1996). By using different techniques, the enzyme has previously been shown to be present in the chick ectodermal epithelium (Tuan & Zrike, 1978; Rieder et al. 1980; Anderson et al. 1981; Tuan, 1984), but its cell-type specific expression in this heterogeneous cell population has not been defined. Two different cell types have been identified in the CAM ectodermal epithelium: the villus cavity cells and the capillary covering cells; likewise, in the endodermal epithelium, basal cells, granule cells, and mitochondria-rich cells have been described (Coleman & Terepka, 1972).

Our aim is to determine unique features of these individual cell types in order to gain insight into their functions in CAM-mediated exchange between the developing embryo and the environment. As a preliminary step, we have further defined the developmental expression and distribution of carbonic anhydrase in the chick CAM by either the histochemical visualisation of the enzyme catalytic sites and the immunohistochemical identification of the CA isoenzymes. Our results indicate that CA of the CAM is not solely an ectodermal enzyme. Indeed, this enzyme accumulated in specific cell types in both the ectodermal and the endodermal epithelium.

MATERIALS AND METHODS

Fertilised eggs of Cobb hens were provided from a commercial source (Garbini, Castelplanio, AN, Italy) and incubated at 99.5 °F in a humidified egg incubator.

Histochemistry

Beginning from incubation d 8 (E 8) to hatching time (E 20–21), fragments of the chorioallantoic membrane, with and without the shell membrane, were removed daily and fixed immediately in a 4% paraformaldehyde and 0.5% glutaraldehyde solution in 0.13 M Millonig buffer (0.16 M NaH_2PO_4 and 0.63 M NaOH), pH 7.3, for 2 h at 4 °C. After washing in the above buffer, small fragments of tissue were embedded in the hydrophilic JB-4 resin (Polysciences, Warrington, PA) according to the manufacturer's instructions. The cobalt-phosphate precipitation method for visualisation of CA activity (Hansson, 1967) was applied to semithin sections (2 μm), as previously detailed (Gabrielli et al. 1990), followed by counterstaining with toluidine blue. For a better interpretation of the histochemical patterns, adjacent sections from the same CAM blocks were stained with toluidine blue. All sections were dehydrated and mounted in Eukitt.

Controls were performed by adding 1×10^{-6} M acetazolamide, a specific CA inhibitor, to the incubation medium or by omitting the sodium bicarbonate substrate.

Antibodies

A specific polyclonal antibody, raised in rabbits against the cytosolic CA isoform purified from chicken erythrocytes (CAII), was kindly provided by Dr P. Linser (Whitney Laboratory, University of Florida, St Augustine, FL). A rabbit antiserum to CAIII from chicken leg muscle was a generous gift from Dr N. Carter (St George's Hospital Medical School, London). The specificity of the antisera has previously been demonstrated (Linser & Moscona, 1981; Jeffery et al. 1982).

SDS-PAGE and Western blot analyses

Fresh CAM samples from 17-d-old embryos were homogenised in 2 volumes of 50 mM Tris-HCl buffer, pH 7.5, and centrifuged at 20000 *g* for 30 min at 4 °C. The supernatants from all samples were assayed for protein with a Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). Aliquots of purified chicken CAII (20 μl) and soluble CAM proteins (30 μg) were subjected to SDS-PAGE under non-reducing conditions according to Laemmli (1970) in a Mini-Protean unit (Bio-Rad) at a constant voltage of 200 V for 40 min. The low molecular weight protein standards were from Bio-Rad. The proteins were transferred from the gels to a nitrocellulose sheet using a constant voltage of 60 V for 90 min in a Bio-Rad Mini Trans-Blot apparatus. After transblotting, the gels were stained with Coomassie Blue to check for complete transfer. The standard lane was cut out and stained with Amido Black. The sample lanes were first incubated with 1% BSA (bovine serum albumin, Sigma, St Louis, MO) in TTBS (0.05% Tween-20 in Tris buffered saline) for 30 min and then with the antiserum to chicken CAII (1:5,000 in TTBS) or the antiserum to chicken CAIII (1:4,000 in TTBS) for 2 h at room temperature. After washing in TTBS (3 times, 10 min each), the lanes were treated with biotinylated goat antirabbit IgG, diluted 1:2000 in TTBS, for 45 min at room temperature and then rinsed 3 times in TTBS, as above. Finally, the avidin-biotin-peroxidase

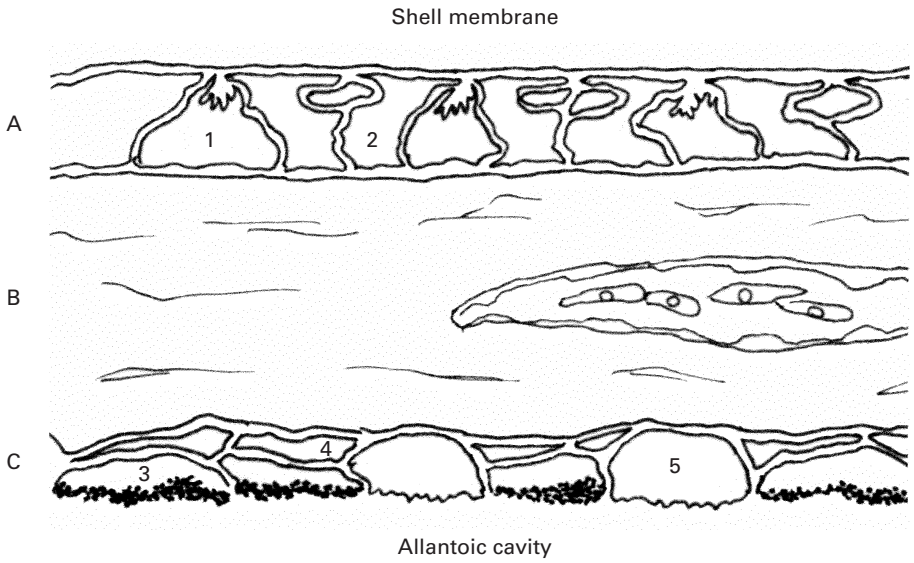

Fig. 1. Schematic representation of the chick CAM after d 12 of incubation. (*A*) In the ectodermal epithelium, villus cavity cells (1) are interspersed among capillary covering cells (2). (*B*) The intermediate mesodermal layer is derived from the fusion of the mesodermal components of chorion and allantois. (*C*) The endodermal (allantoic) epithelium is composed of granule cells (3), basal cells (4), and mitochondria-rich cells (5).

complex, diluted 1:1,000 in TTBS, was applied for 30 min, followed by washing as above. Signals were detected by using the 3-3′ diaminobenzidine chromogen (DAB/substrate kit) for 8 min at room temperature. All reagents were from Vector Laboratories (Burlingame, CA).

Control Western blots were carried out using rabbit preimmune serum instead of the primary antibodies.

Immunohistochemistry

Fragments of the chick CAM, removed daily from E 8 to hatching, were fixed in Bouin's solution for 3 h, dehydrated through graded ethanols, cleared in xylene and embedded in paraffin wax at 56–58 °C. Sections (5 μm), mounted on gelatin coated slides, were rehydrated and treated according to the avidin-biotin-peroxidase system, using reagents from Vector Laboratories. Inactivation of endogenous peroxidase was obtained with 0.3% H_2O_2 in methanol for 30 min, followed by application of the avidin-biotin blocking kit. Sections were incubated for 20 min with normal goat serum diluted 1:5 with phosphate buffered saline (PBS) plus 1% BSA. The sections were then treated with antichicken CAII (1:5,000) or antichicken CAIII (1:4,000) overnight at room temperature in a humidified chamber. Appropriate biotinylated secondary antibody was used for 45 min, followed by avidin-biotin-peroxidase complex (45 min). Peroxidase activity sites were visualised using the DAB/substrate kit. Unless otherwise specified, dilutions were made with PBS. At any step of the immunohistochemical procedure, sections were rinsed in 3 changes of PBS for 5 min each.

Controls were performed by substituting the primary antibodies with PBS/BSA or with rabbit preimmune serum. As an additional control, some sections were incubated with the primary antibody that had been pretreated for 24 h at 4 °C with 0.2 mg/ml of the relative antigen, and subsequently subjected to the remaining steps of the immunohistochemical sequence.

RESULTS

The layering of the chick CAM and the structural features of the different cell types in its epithelia are illustrated schematically in Figure 1. The ectodermal (chorionic) epithelium lies adjacent to the shell membrane and is involved in gaseous exchange and calcium mobilisation and transport (Tuan, 1987). It is composed of 2 major cell types: the villus cavity (VC) cells, characterised by an apical depression lined by numerous microvilli, and the capillary covering (CC) cells, provided with long thin cytoplasmic processes which overlie the intrachorionic sinus (Coleman & Terepka, 1972). The endodermal (allantoic) epithelium lines the allantoic cavity and regulates water and electrolyte transport from its lumen (Simkiss, 1980). Three different cell types, basal cells, granule cells, and mitochondria-rich (MR) cells, can be distinguished on the basis of their size, position through the epithelium, and content of cytoplasmic granules.

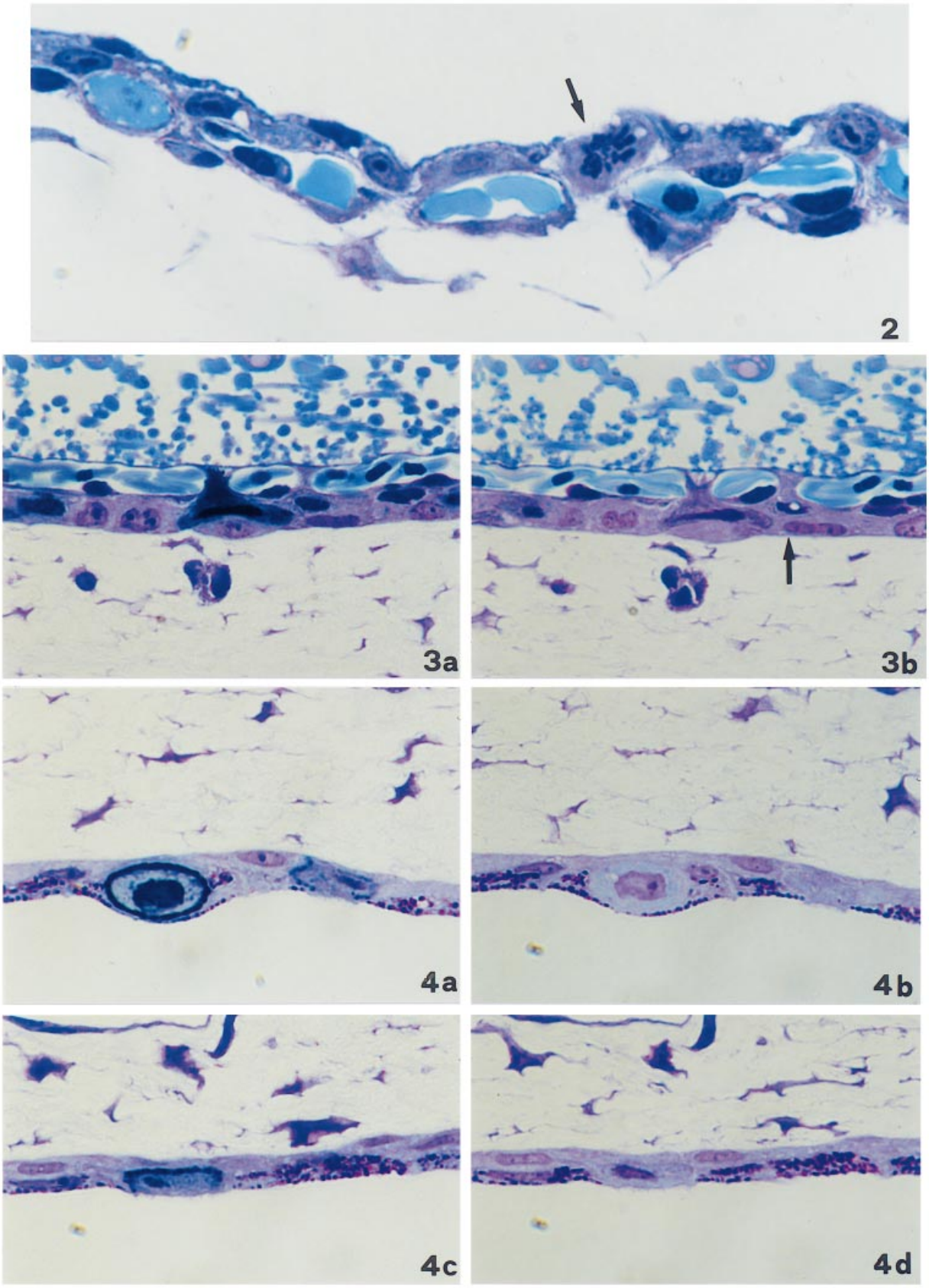

Fig. 2. Ectodermal layer of the chick CAM, removed without shell membrane, at E 8. The histochemical method for CA demonstration produces no reaction product in the still differentiating epithelium. Arrow, mitotic figure. Toluidine blue counterstaining. $\times 1100$.

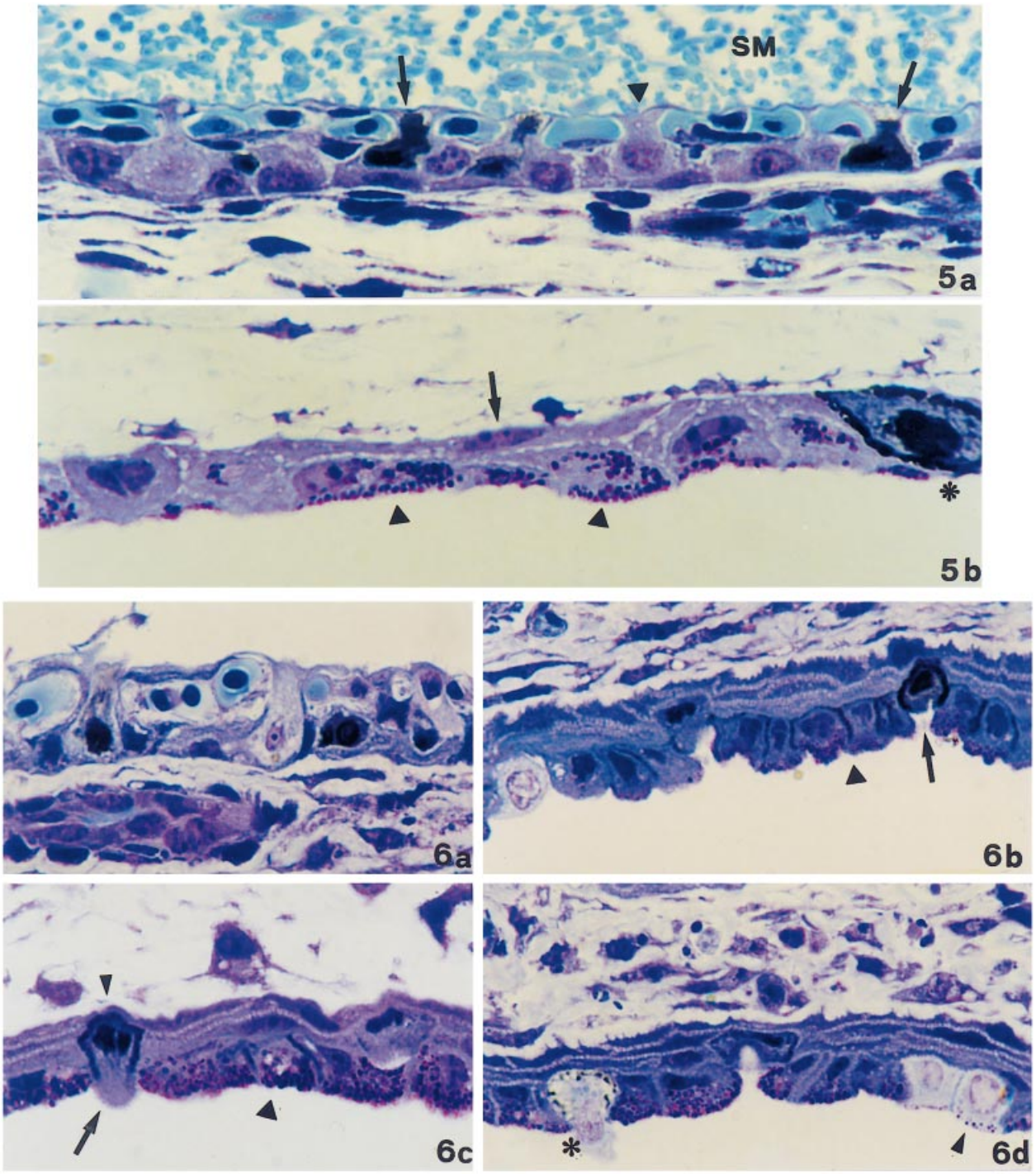

Fig. 3. (*a*, *b*) Adjacent sections of the CAM ectodermal epithelium at E 13. One section has been subjected to the histochemical method for CA demonstration followed by toluidine blue counterstaining (*a*), the other to toluidine blue staining (*b*). (*a*) CA activity produces a dark reaction product localised in a unique cell. (*b*) The CA positive cell can be identified as a VC cell by the pronounced eosinophilic staining of its cytoplasm and by characteristic cytoplasmic protrusions into the apical depression. A typical CC cell (arrow) can be also recognised; it shows a lighter cytoplasm which extends apically towards the shell membrane. Comparison with *a* shows that the CC cell lacks CA activity. × 1100.

Fig. 4. (*a–d*) Cellular localisation of CA activity in the CAM endodermal epithelium at E 13, comparing adjacent sections stained with toluidine blue following application of the histochemical method for CA (*a*, *c*) and without preliminary treatment (*b*, *d*). (*a*) CA activity can be detected at the cell membranes and nucleus of cells which can be identified as MR cells on the basis of their size, position through the epithelium, and absence of cytoplasmic granules (*b*). (*c*, *d*) Appropriate sectioning of the epithelium shows that CA activity associated with the MR cell membranes is restricted to the basolateral membranes. The apical cell membrane, lining the allantoic cavity, is unstained. × 1100.

[*continued overleaf*]

Neither formation of the reaction product in the histochemical approach nor penetration of the antibodies in the immunohistochemical approach were hampered in the tissue removed with shell membrane. In both techniques, no appreciable differences in the staining pattern were detected in the presence or absence of shell membrane.

Histochemistry

The developmental patterns of the chick CAM showed early evidence of distinct cell lineages in both epithelial layers. At E 8, the ectodermal epithelium appeared to be bilayered, not yet covered by the developing superficial blood sinus. At this stage, the histochemical reaction for the demonstration of CA produced no staining (Fig. 2). The enzyme activity first appeared either in ectodermal or endodermal epithelium at E 11–12, concurrent with the full extension of the CAM around the embryo. A developmentally-regulated increase both in intensity of the histochemical staining and number of the reactive sites was evident up to E 18–19.

In the ectodermal epithelium CA activity was localised in cells which, by comparing the histochemical pattern with an adjacent morphological section, were identified as VC cells (Fig. 3*a*, *b*); very strong staining covered the CA positive VC cells, completely masking the cytoplasmic structures; nuclei were also stained (Fig. 5*a*). The CC cells showed no enzyme activity at any stage examined (Figs 3, 5*a*). In the final days of incubation, a gradual reduction in the VC cell staining was observed, in parallel with the appearance of regression features in both the ectodermal cell types (Fig. 6*a*).

In the endodermal epithelium, active CA was limited to MR cells, scattered among the more numerous, unstained basal cells and granule cells (Figs 4, 5*b*). The enzyme activity produced granular staining in the cytoplasm and heavy deposits of the reaction product at cell membranes and nucleus (Figs 4*a*, 5*b*). The membrane-associated CA activity proved to be confined to the basolateral cell membranes, without extension to the apical membrane (Fig. 4*c*, *d*). During the final days of incubation, together with the regressive modifications of the endodermal epithelium, a distinct pattern of CA staining emerged (Fig. 6*b–d*); besides persisting in sometimes largely modified MR cells, transient expression of the enzyme activity was detected at the basolateral membranes of granule cells (Fig. 6*b*) which were undergoing a progressive degranulation and exhibited metachromatic staining of their residual granules (Fig. 6*c*). The occurrence of light cells, often protruding into the allantoic cavity, was an additional feature characteristic of the tissue degeneration (Fig. 6*b*, *d*).

Omitting the sodium bicarbonate substrate or the addition of the specific CA inhibitor, acetazolamide, to the incubation medium blocked the production of reaction product, indicating that the histochemical staining was highly specific for CA activity.

Western blot analyses

Immunoblotting analysis of soluble proteins from CAM extracts with anti-CAII revealed a major immunoreactive band with the same molecular mass as CAII purified from chicken erythrocytes (Fig. 7). Faint minor bands that were also detected probably represent fragments due to proteolytic degradation of the CAII polypeptide. The anti-CAIII sera showed no immunoreactivity with blotted CAM proteins. In addition, control blots with preimmune serum were negative.

Immunohistochemistry

At early embryogenic stages, CA was not detected in the developing CAM by immunohistochemistry (Fig. 8). From E 11–12 up to hatching, intense cell type-specific immunostaining was observed using the antiserum directed against avian CAII (Figs 9–12). This immunostaining fully reproduced the distribution of CA activity visualised by the histochemical technique. Only VC cells in the ectodermal layer and MR cells in the endodermal layer were strongly stained. Immunostaining was diffusely distributed over the entire cytoplasm of the reactive cells (Figs

Fig. 5. (*a*, *b*) Chick CAM at E 14. In the ectodermal epithelium (*a*), facing the shell membrane (SM), CA activity is located only in VC cells (arrows). The CC cells are unstained (arrowhead). In the allantoic epithelium (*b*), a strongly CA positive MR cell (asterisk) and a negative MR cell (at the left corner) can be observed. No enzyme activity is present in the granule cells (arrowheads) and basal cells (arrow). Histochemical method for CA demonstration and toluidine Blue counterstaining. × 1100.

Fig. 6. (*a–d*) Histochemical demonstration of CA in degenerating CAM at E 20. (*a*) The ectodermal epithelium shows no sites of CA activity. (*b*, *c*, *d*) The endodermal epithelium is highly stratified; the reaction product, besides persisting in MR cells (arrows in *b*, *c*), appears at the basolateral membranes of granule cells (arrowheads in *b*, *c*). Occurrence of light cells, showing either apical protrusions into the allantoic cavity (asterisk in *d*) or metachromatic granules scattered at the apical cell portion (arrowhead in *d*), can be observed. Histochemical method for CA demonstration and toluidine blue counterstaining. × 1100.

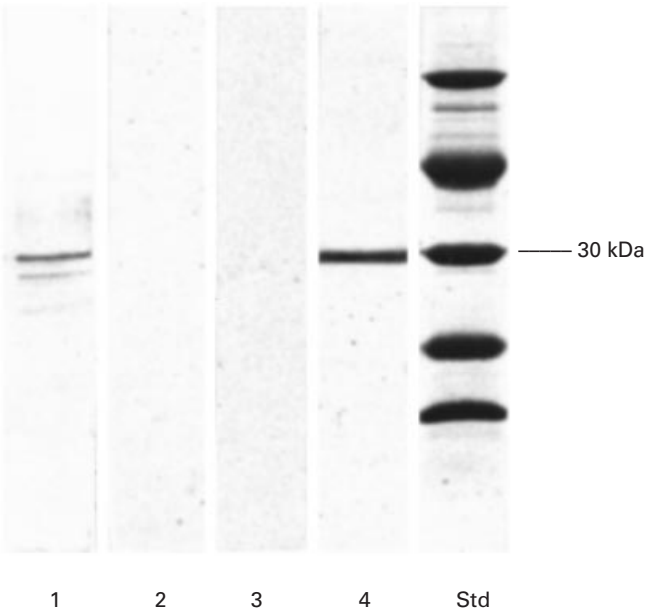

Fig. 7. Western blot analyses of soluble proteins from CAM extracts using anti-CAII (lane 1), anti-CAIII (lane 2), and preimmune serum (lane 3). Immunoblotting of purified chicken CAII with anti-CAII confirms the specific reactivity of the antiserum used in this work (lane 4). Std, molecular weight standard. The molecular mass of bovine CA (30 kDa) is marked.

9–11). During the final days of incubation, the expression pattern of CAII showed marked modifications, mainly at the endodermal epithelium where a common finding was the occurrence of strongly stained MR cells (Fig. 11), often very enlarged or elongated apically towards the allantoic cavity (Fig. 12).

Similar studies with the antibody to chicken CAIII produced no immunostaining at any stage examined. All control sections, incubated with PBS/BSA or preimmune serum, also failed to reveal any staining. In addition, controls performed using the primary antibody, following preincubation with CAII antigen, resulted in no detectable immunostaining (Fig. 10*b*).

DISCUSSION

Since the first identification of CA activity in the chick CAM (Tuan & Zrike, 1978), much evidence from biochemical (Tuan, 1984; Tuan et al. 1986) and inhibition studies (Tuan, 1980) have suggested an involvement of this enzyme in CAM-mediated processes that take place during chick embryogenesis. By means of different techniques, evidence of carbonic anhydrase localisation in the CAM ectodermal epithelium was also provided. Histochemical and immunohistochemical reports documented a uniform distribution of the enzyme throughout the ectodermal layer (Tuan et al. 1978; Tuan, 1984) but cell preservation and morphological resolution were inadequate for distinguishing cell types. On the other hand, labelled inhibitor autoradiography as well as immunoelectron microscopy indicated that there was a moderate amount of CA in CC cells and very high levels in VC cells (Rieder et al. 1980; Anderson et al. 1981). Thus, despite the aforementioned findings supporting its presence in the CAM ectodermal epithelium, the precise cellular distribution and isoform expression patterns of CA in the chick CAM have yet to be firmly established. To further elucidate the significance of this enzyme in CAM-mediated processes and to further characterise the cell populations of the CAM epithelia, we have determined the CA distribution patterns in the developing chick CAM by histochemical and immunohistochemical techniques. Application of the histochemical cobalt precipitation method has been performed with slight modifications (Ridderstrale, 1991) on tissue embedded in JB-4 hydrophilic resin which combines the advantages of good retention of the enzyme activity and high resolution of the morphological details. In addition, the occurrence of the known cytosolic isoforms of the avian carbonic anhydrase, CAII and CAIII, was investigated using specific polyclonal antibodies.

The results of the 2 technical approaches were consistent in that they indicated a similar profile for the developmentally-regulated expression of carbonic anhydrase in the CAM epithelia. The enzyme first appeared in 11–12-d-old embryos, concomitant with the differentiation of distinct cell types (Coleman & Terepka, 1972) and the onset of CAM functional activity (Terepka et al. 1969). The intensity and distribution of CA staining gradually increased during embryogenesis up to the final days of incubation, in agreement with the developmental profile of the enzyme activity from biochemical analyses (Tuan & Zrike, 1978).

In the ectodermal epithelium, the distribution of CA catalytic sites and CAII immunostaining clearly showed that the enzyme was restricted to VC cells. Carbonic anhydrase was never found in CC cells. The histochemistry and immunohistochemistry in conjunction with the Western blot analyses strongly suggest that CA activity of the CAM is mediated by the cytosolic isoenzyme purified from the adult chicken erythrocytes and termed as CAII, in analogy with the corresponding mammalian isoform (Sanyal, 1984). This conclusion is consistent with inhibition studies that tested the effect of sulphonamides on calcium uptake both in intact CAM and in a cell-free microsomal system (Tuan et al. 1986).

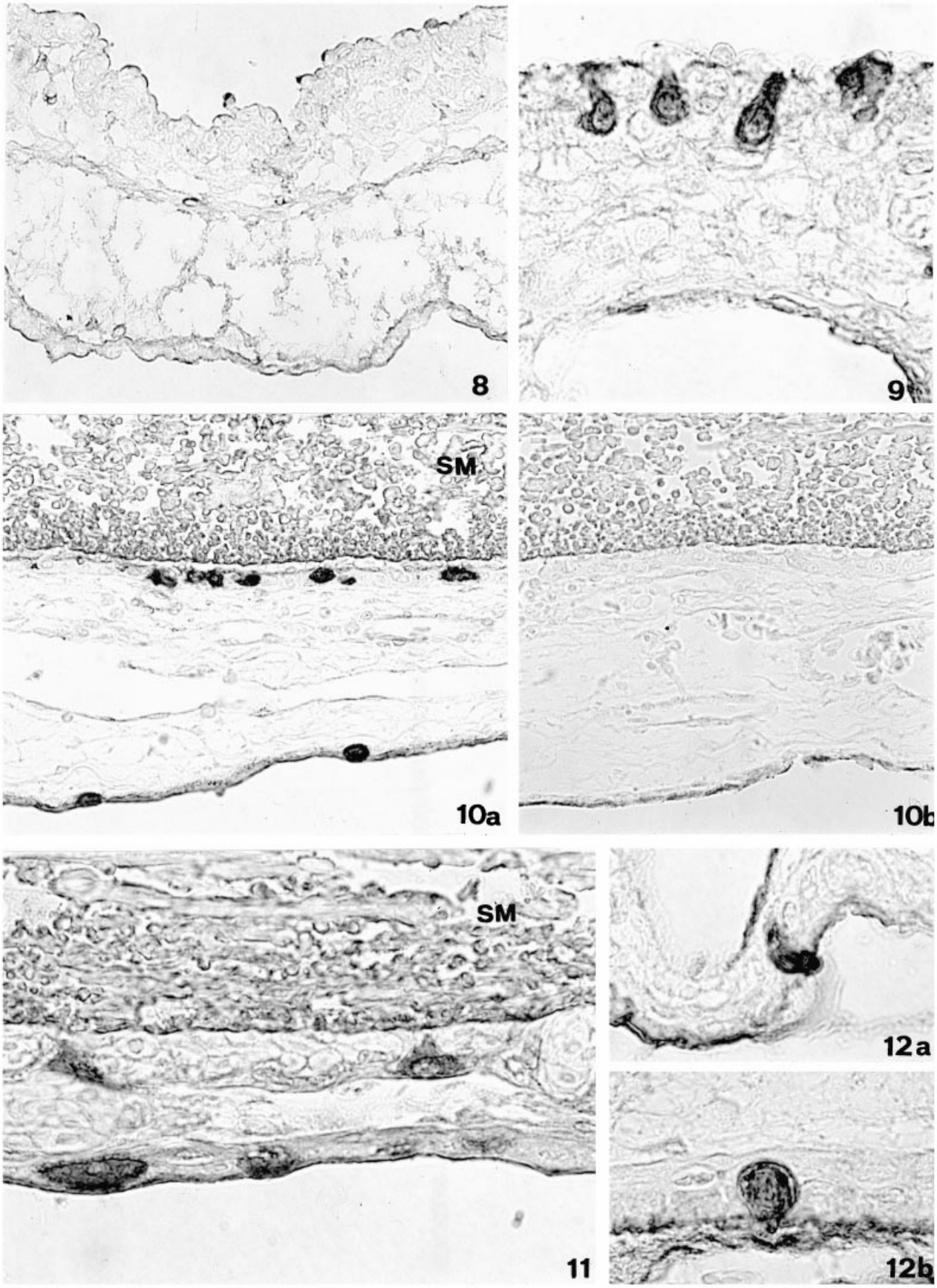

Fig. 8. The chick CAM at E 9 following immunohistochemical demonstration of CAII. No staining can be detected either in the ectodermal or endodermal layers. The tissue has been removed without the shell membrane. × 450.

The intracellular localisation of CA in the VC cells correlates well with the identification in these cells of morphological features characteristic of proton secreting cells as well as with the observation that the vacuolar H^+-ATPase accumulates in the apical membrane and in subapical vesicles in VC cells (Nabaitz et al. 1995). Taken together, these data suggest that VC cells are a specialised chorionic cell type involved in the H^+ secretion that is required for the solubilisation of the shell mineral calcite to produce calcium ready for uptake. The function of VC cells would thus be analogous to other known proton secreting cells, such as the A-type intercalated cells of the renal collecting system (Brown & Breton, 1996). Accordingly, VC cells might also participate in reabsorption of bicarbonate ions that are released together with calcium ions from the eggshell, thus contributing to the gradual increase in plasma bicarbonate through which the chick embryo prevents metabolic acidosis during the second half of development. A contribution to acid-base balance from the kidney has been also suggested (Dawes & Simkiss, 1971). In this view, secretion of acid urine and bicarbonate reabsorption from the renal tubules might be substantiated by the early expression and extensive distribution of carbonic anhydrase in the developing kidney, as recently demonstrated in the quail embryo (Gabrielli et al. 2000).

Our demonstration of carbonic anhydrase in the MR cells in the endodermal epithelium, in addition to the previously reported occurrence of vacuolar H^+-ATPase at their luminal membranes (Narbaitz et al. 1995), supports an involvement of this cell type in the progressive acidification of the allantoic fluid occurring during incubation (Dawes & Simkiss, 1971). By subserving apical extrusion of H^+, probably via the H^+-ATPase pump and/or in exchange with Na^+, carbonic anhydrase of the MR cells might also mediate HCO_3^- reabsorption from the allantoic cavity, that contributes to the ability of the embryo to buffer acid generated by metabolic processes. Evidence for active transport mechanisms by the CAM endodermal epithelium, on the other hand, have been reported elsewhere (Stewart & Terepka, 1969). In particular, active transport of Na^+ is thought to account for the gradual reabsorption from the allantoic fluid of water that is made available by concomitant urate precipitation (Porter, 1963).

Reabsorption of water and ions is substantially reduced by E 18, in parallel with the appearance of degenerative features in the endodermal epithelium. We have observed concurrent changes in the distribution pattern of CA. The enzyme was still expressed in numerous MR cells, characterised by a large size and luminal protrusions. Interestingly, the histochemical technique also visualised CA activity at the basolateral membranes of adjacent granule cells where immunohistochemistry failed to detect any staining. A possible conclusion is that such a late expression pattern of CA activity in the endodermal epithelium depends on an additional CA isoenzyme, distinct from the cytosolic CAII or CAIII. Alternatively, our failure to visualise immunostaining at these sites might be due to a CAII concentration which is too low to be detected by the immunohistochemical technique.

The late occurrence in the endodermal epithelium of large, probably degenerating cells which showed light cytoplasm and metachromatic granules scattered at the apical cell portion, requires further study.

In summary, the cell type-specific expression of CA in the chick CAM provides additional data regarding the potential function of specific cell types in both the ectodermal and the endodermal epithelium. Through the differentiation of these highly specialised epithelial cells, the relatively simple structure of the CAM is able to mediate multiple important functions during chick embryogenesis.

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Fig. 9. At E 12, in the ectodermal epithelium of the chick CAM strongly reactive cells can be observed following immunostaining with antiserum to CAII. × 1100.

Fig. 10. (*a*, *b*) The chick CAM, with the shell membrane (SM), at E 17. The specificity of CAII immunostaining (*a*) in the ectodermal and endodermal epithelium is confirmed by comparison with the negative control (*b*) obtained using anti-CAII preincubated with the relative antigen (0.2 mg/ml) for 24 h. × 450.

Fig. 11. In the chick CAM at E 19, marked CAII immunostaining is still present in both ectodermal VC cells and endodermal MR cells. SM, shell membrane. × 1100.

Fig. 12. (*a*, *b*) In the degenerating endodermal epithelium of the chick CAM (E 20), CAII immunohistochemistry results in strong staining of MR cells which often exhibit apical protrusions into the allantoic cavity (*a*) or very large size (*b*). No CAII staining is detectable in granule and basal cells. × 1100.

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REFERENCES

ANDERSON RE, GAY CV, SCHRAER H (1981) Ultrastructural localization of carbonic anhydrase in the chorioallantoic membrane by immunocytochemistry. *Journal of Histochemistry and Cytochemistry* **29**, 1121–1127.

AUSPRUNK DH (1986) Distribution of hyaluronic acid and sulfated glycosaminoglycans during blood-vessel development in the chick chorioallantoic membrane. *American Journal of Anatomy* **177**, 313–331.

BROWN D, BRETON S (1996) Mitochondria-rich, proton-secreting epithelial cells. *Journal of Experimental Biology* **199**, 2345–2358.

CARTER MJ (1972) Carbonic anhydrase: isoenzymes, properties, distribution, and functional significance. *Biological Reviews* **47**, 465–513.

COLEMAN JR, TEREPKA AR (1972) Fine structural changes associated with the onset of calcium, sodium and water transport by the chicken chorioallantoic membrane. *Journal of Membrane Biology* **7**, 11–127.

DAWES CM, SIMKISS K (1969) The acid-base status of the blood of the developing chick embryo. *Journal of Experimental Biology* **50**, 79–86.

DAWES CM, SIMKISS K (1971) The effects of respiratory acidosis in the chick embryo. *Journal of Experimental Biology* **55**, 77.

FREEMAN BM, VINCE MA (1974) Gaseous exchange and oxygenation of the embryo. In *Development of the Avian Embryo. A Behavioural and Physiological Study*, pp. 119–159. London: Chapman and Hall.

GABRIELLI MG, PALATRONI P, VINCENZETTI S (1990) Renal carbonic anhydrase in the quail *Coturnix coturnix japonica*. I. Activity and distribution in male and female metanephros. *Histochemical Journal* **22**, 579–587.

GABRIELLI MG, MATERAZZI G, MENGHI G (2000) The metanephros of the quail embryo. Developmental expression of carbonic anhydrase investigated by multiple approaches. *Journal of Anatomy* **196**, 31–40.

HANSSON HPJ (1967) Histochemical demonstration of carbonic anhydrase activity. *Histochemie* **11**, 112–128.

JEFFERY D, EDWARDS YH, JACKSON MJ, JEFFERY S, CARTER ND (1982) Zinc and carbonic anhydrase. III. Distribution in mammalian muscle. *Comparative Biochemistry and Physiology* **B 73**, 971–975.

LAEMMLI UK (1970) Cleavage and structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

LEESON RS, LEESON CR (1963) The chorioallantois of the chick. Light and electron microscope observations at various times of incubation. *Journal of Anatomy* **97**, 585–595.

LINSER PJ, MOSCONA AA (1981) Carbonic anhydrase C in the neural retina: transition from generalized to glia specific cell localization during embryonic development. *Proceedings of the National Academy of Sciences of the USA* **76**, 6476–6480.

MAREN TH (1967) Carbonic anhydrase: chemistry, physiology and inhibition. *Physiological Reviews* **47**, 595–781.

NARBAITZ R (1977) Structure of the intra-chorionic blood sinus in the chick embryo. *Journal of Anatomy* **124**, 347–354.

NARBAITZ R, BASTANI B, GALVIN NJ, KAPAL VK, LEVINE DZ (1995) Ultrastructural and immunocytochemical evidence for the presence of polarized plasma membrane H^+-ATPase in two specialized cell types in the chick embryo chorioallantoic membrane. *Journal of Anatomy* **186**, 245–252.

PACKARD MJ, PACKARD GC (1984) Comparative aspects of calcium metabolism in embryonic reptiles and birds. In *Respiration and Metabolism of Embryonic Vertebrates* (ed. Seymour RS), pp. 155–177. Dordrecht: W. Junk.

PARKILLA S, PARKILLA A-K (1996) Carbonic anhydrase in the alimentary tract. Roles of the different isoenzymes and salivary factors in the maintenance of optimal conditions in the gastrointestinal canal. *Scandinavian Journal of Gastroenterology* **31**, 305–317.

PORTER P (1963) Physico-chemical factors involved in urate calculus formation. II. Colloidal flocculation. *Research in Veterinary Sciences* **4**, 592–602.

RIDDERSTRALE Y (1991) Localization of carbonic anhydrase by chemical reactions. In *The Carbonic Anhydrases. Cellular Physiology and Molecular Genetics* (ed. Dodgson SJ, , Tashian RE, , Gros G, Carter ND), pp. 134–144. New York, London: Plenum Press.

RIEDER E, GAY CV, SCHRAER H (1980) Autoradiographic localization of carbonic anhydrase in the developing chorioallantoic membrane. *Anatomy & Embryology* **159**, 17–31.

SANYAL G (1984) Comparative carbon dioxide hydration kinetics and inhibition of carbonic anhydrase isoenzymes in vertebrates. *Annals of the New York Academy of Sciences* **429**, 165–178.

SIMKISS K (1980) Water and ionic fluxes inside the egg. *American Zoology* **20**, 384–393.

SLY WS, HU PY (1995) Human carbonic anhydrases and carbonic anhydrase deficiencies. *Annual Reviews of Biochemistry* **64**, 375–401.

STEWART ME, TEREPKA AR (1969) Transport functions of the chick chorioallantoic membrane. I. Normal histology and evidence for active transport from the allantoic fluid, *in vivo*. *Experimental Cell Research* **58**, 93–106.

TEREPKA AR, STEWART ME, MERKEL N (1969) Transport functions of the chick chorio-allantoic membrane. II. Active calcium transport, *in vitro*. *Experimental Cell Research* **58**, 107–117.

TUAN RS (1980) Calcium transport and related functions in the chorioallantoic membrane of cultured shell-less chick embryos. *Developmental Biology* **74**, 196–204.

TUAN RS (1984) Carbonic anhydrase and calcium transport function of the chick embryonic chorioallantoic membrane. *Annals of the New York Academy of Sciences* **429**, 459–472.

TUAN RS (1987) Mechanisms of regulation of calcium transport by the chick chorioallantoic membrane. *Journal of Experimental Zoology*, suppl. **1**, 1–13.

TUAN RS, ZRIKE J (1978) Functional involvement of carbonic anhydrase in calcium transport of the chick chorioallantoic membrane. *Biochemical Journal* **176**, 67–74.

TUAN RS, CARSON MJ, JOZEFIAK JA, KNOWLES KA, SHOTWELL BA (1986) Calcium-transport function of the chick embryonic chorioallantoic membrane. II. Functional involvement of calcium-binding protein, Ca^{2+}-ATPase and carbonic anhydrase. *Journal of Cell Science* **82**, 85–97.