Int. J. Dev. Biol. 39: 663-666 (1995)

Short Contribution

Differential spatiotemporal expression of E- and P-cadherin during mouse tooth development

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ABSTRACT Changes in E- and P-cadherin (E- and P-CD) expression during embryonic mouse first molar development were analyzed by immunohistochemistry. During the induction and morphogenesis stages (bud, cap and early bell stages), E-CD was expressed in the cells of the invaginating epithelial tooth bud and in the cells of the outer enamel epithelium, stellate reticulum and stratum intermedium, suggesting a role for this molecule in the maintenance of enamel organ architecture. On the other hand, P-CD was strongly expressed in the inner enamel epithelium suggesting its participation in the processes of mesenchymal induction. During the cytodifferentiation stage (late bell stage), E-CD was expressed in polarizing preameloblasts, but cadherin expression was restricted to the basal and apical poles of differentiated secretory ameloblasts, where the zonula adherens type of cell-cell junctions is located. The present study demonstrates for the first time the spatiotemporal expression of cadherins during tooth development and suggests differential and specific roles for E-CD and P-CD during the morphogenesis and cytodifferentiation processes of this organ.

KEY WORDS: *E-cadherin, P-cadherin, tooth morphogenesis, adhesion*

Cadherins are a superfamily of Ca^{2+} dependent cell-cell adhesion molecules which are expressed in most kinds of vertebrate tissues and play a critical role in morphogenesis as well as in the maintenance of adult tissue architecture (Takeichi, 1991). E- and P-cadherin are glycoproteins which share a common basic structure but differ in tissue distribution and immunological properties (Geiger and Ayalon, 1992; Grunwald, 1993). In certain cases, more than one cadherin is coexpressed in a tissue during development. For example, E- and P-cadherin are coexpressed in metanephric tubules, the eye, inner ear, and epidermis during mouse development (Nose and Takeichi, 1986; Takeichi, 1988; Hirai *et al.*, 1989). Those studies also showed that E- and P-CD display a unique spatiotemporal expression pattern closely related with morphogenetic events.

In order to further examine the tissue specificity and the morphogenetic role of E- and P-CD expression, we have chosen the developing mouse molar since its development takes place in several precise stages (Lumsden, 1988) and, in addition, the embryonic tooth constitutes an accurate model for the analysis of epithelial-mesenchymal interactions (Thesleff *et al.*, 1989; Vainio *et al.*, 1993; Ruch *et al.*, 1995; Thesleff *et al.*, 1995; Zeichner-David *et al.*, 1995). We have analyzed the expression of both adhesion molecules during the stages of induction (bud stage), morphogenesis (cap and early bell stages) and cytodifferentiation (late bell stage) in the first molar by immunohistochemistry.

During the bud stage (12- and 13-day embryos) all the epithelial cells in the presumptive enamel organ expressed E-CD (Fig. 1A), but P-CD immunoreactivity was only present in the cells of the basal layer, the presumptive inner enamel epithelium (Fig. 1B). These basal cells not only have the greater proliferating activity among epithelial cells of the tooth germ (Vaahtokari *et al.*, 1991), but are also able to induce the mesenchyme to condense around the epithelial bud (Lumsden, 1988). Present findings could indicate that the expression of P-CD is associated with the proliferating activity of cells and with their ability to induce the mesenchyme, as previously suggested in the epidermis (Hirai *et al.*, 1989). In the skin, P-CD is expressed in the basal layer and in the hair matrix cells, which are known to have the highest mitotic activity in the epidermis. In addition, the inhibition of P-CD with antibodies in skin organ cultures not only destroyed the columnar arrangement of basal cells but also suppressed the condensation of dermal cells (which do not expressed P-CD) around the outer root sheath during the development of hair follicles (Hirai *et al.*, 1989).

During mouse tooth development, from the cap stage until the late bell stage (14- day embryos to 3-day postnatal mice), E-CD was strongly expressed in the cells of the dental lamina, outer enamel epithelium, stellate reticulum and stratum intermedium

Abbreviations used in this paper: E-CD, E-cadherin; P-CD, P-cadherin.

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0214-6282/95/$03.00

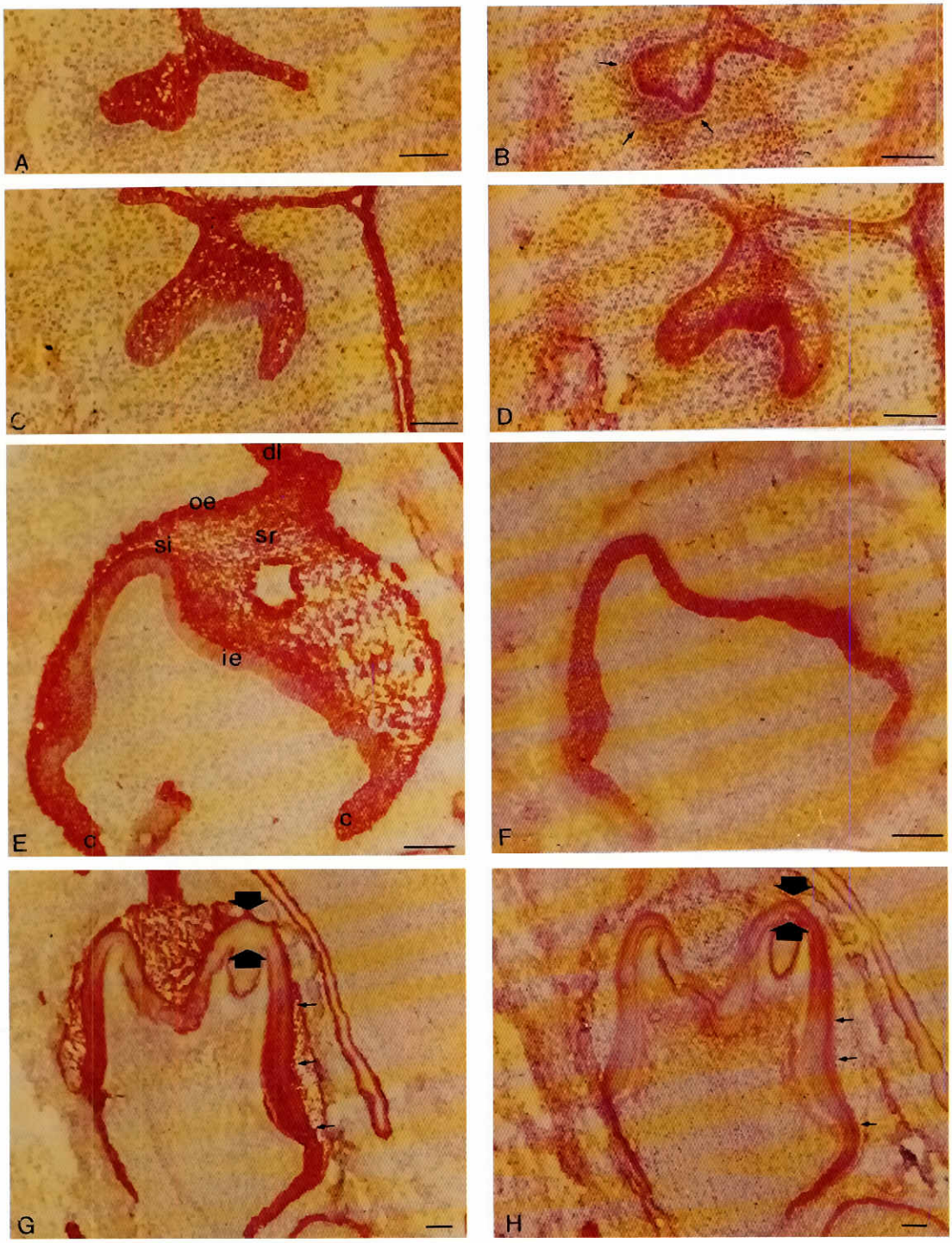

Fig. 1. Comparison between E- and P-CD expression in the first molar from bud until late bell stage of development. (A and B) *Molar tooth germ at bud stage (13-day embryo). E-CD is expressed in all cells of the bud tooth* **(A)**, *but P-CD expression is restricted to the basal cells overlying condensed mesenchymal cells (arrows)* **(B). (C to F)** *In the cap (15-day embryo) and early bell stage (17-day embryo), E-CD is expressed in the cells of the dental lamina, outer enamel epithelium, stellate reticulum, stratum intermedium and cervical loops* **(C,E)**, *whereas, inner enamel epithelium expressed P-CD* **(D and F). (G and H)** *In late bell stage (1-day postnatal mouse), polarizing ameloblasts (arrows) expressed E-CD around the entire cytoplasm* **(G)**, *but P-CD expression is restricted to the proximal pole* **(H)**. *In the more developed cuspal areas, polarized ameloblasts expressed P-CD at their proximal and distal poles. The dental lamina, outer enamel epithelium, stellate reticulum, stratum intermedium and cervical loops also expressed E-CD in this stage. Dental lamina (dl), outer enamel epithelium (oe), stratum intermedium (si), stellate reticulum (sr), inner enamel epithelium (ie), cervical loop (c). Original magnification, 10X in A-F, and 4X in G and H. Bars, 50 μm.*

(Fig. 1C,1E and 1H) suggesting a role for this molecule in maintaining the structure of the enamel organ. In contrast, the expression of P-CD was mostly restricted to the inner enamel epithelium (Fig. 1D,1F and 1G). In addition, the cells of the inner enamel epithelium had a pattern of E- and P-CD expression that changed dynamically with development in association with morphogenetic and cytodifferentiation events. During the cap and early bell stages (14-17 day embryos), the inner enamel epithelium mainly expressed P-CD (Fig. 1B and 1F), but coexpression of E- and P-CD was present at the cervical loops (Fig. 1E and 1F). The morphogenesis of the tooth occurs in these stages that are characterized by rapid cell proliferation and establishment of the morphology of the tooth crown. The movement of the apposed epithelial and mesenchymal layers generate a spatial configuration at their interface which varies with tooth position, being spatulate or conical in the case of incisors or canines and folded into a more complicated shape of elevations and depressions in the case of the molars (Lumsden, 1988). The folding of epithelial sheets is another type of cadherin-mediated morphogenetic process, which occurs, for example, during the formation of the neural tube or lens (Geiger and Ayalon, 1992). The strong P-CD expression observed in the inner enamel epithelium during these stages suggests that P-CD could be involved in the morphogenetic process of epithelial folding in the enamel organ.

In the late bell stage (18-19-day embryos, and 1-day postnatal mice), proliferating epithelial cells adjacent to cervical loops continued to coexpress E- and P-CD (Fig. 1G and 1H). Once odontoblasts were polarized and secreted dentin matrix, preameloblasts started to polarize. Polarizing preameloblasts expressed E-CD all over the cell surface (Fig. 1G) but P-CD was only found in the proximal (basal) end (Fig. 1H). In the cuspal areas, polarized ameloblasts only expressed cadherins at their proximal and distal surfaces, P-CD being expressed in both poles of the cells (Fig. 1H) whereas E-CD becomes restricted to the proximal end in the more developed cusps (Fig. 1G). The sequential pattern of cadherin expression along the inner enamel epithelium suggests a differential role for E- and P-CD during ameloblast cytodifferentiation: E-CD would participate in cell polarization, while both types of cadherins would maintain the cohesion of differentiated ameloblasts. The acquisition of a polarized phenotype, which is important in the generation of vectorial functions such as secretion and absorption, is a multistage process which requires extracellular signals and the organization of the proteins in the cytoplasm and on the plasma membrane (Rodriguez-Boulan and Nelson, 1989). Several studies have implicated E-CD in the polarization of epithelial cells (Maars *et al.*, 1993) through its participation in the formation and maintenance of junctional complex (Gumbiner, 1990). Immunolocalization studies based on electron microscopy (Boller *et al.*, 1985) have demonstrated the specific association of E-CD with the zonula adherens junctions of the junctional complex. In the adherens junctions, cadherin molecules act as adhesion molecules and actin filaments are densely associated with the plasma membrane through a well- developed plasmalemmal undercoat. Although the precise molecular organization of the adherens junctions is under study, the molecular linkage between cadherins and actin filaments seems to be mediated by cadherin-associated (catenins) and actin-associated (alpha-actinin, vinculin, etc.) proteins (Tsukita *et al.*, 1992). The pattern of cadherin expression observed in the bell stage of tooth development is similar to those reported for actin, alpha-actinin and vinculin (Kluber *et al.*, 1988): these molecules are expressed around the entire peripheral cytoplasm of the polarizing preameloblasts but, when ameloblasts reach polarization and functional differentiation, they accumulate at the proximal and distal poles of the cells, where the junctional complex are located (Matthiessen and Møllgård, 1973).

In summary, the present study demonstrates that E- and P-CD expression is regulated in a distinct spatiotemporal pattern during tooth development, suggesting differential and specific roles for E-CD and P-CD during morphogenesis and cytodifferentiation processes. Since developing tooth is accessible to tissue recombination studies for the analysis of epithelial-mesenchymal interactions, and terminal ameloblast and odontoblast differentiation occurs in organ cultures, the developing tooth provides an adequate model to further study the mechanisms by which cadherin expression is regulated during morphogenesis and cytodifferentiation.

Experimental Procedures

Preparation of the specimens

Mice, strain BALB/c, were mated overnight and the day of finding a vaginal plug designated day zero. At embryonic days 13-19, mothers were killed by ether overdose and the uteri and embryos aseptically removed. Embryos and postnatal mice 1-day-old were decapitated and the heads embedded in Tissue-Tek O.C.T. compound (Miles Laboratory, Naperville, IL, USA), snap-frozen in liquid nitrogen-cooled isopentane, and sectioned or stored at -70°C. Cryostat 5-6 µm thick coronal head sections were cut, fixed in 10% formaline in Tris-buffer containing 10 mmol/L Ca^{2+}, pH 7.2 for 3 min and postfixed at -20°C in methanol for 1 min and in acetone for 3 min. After washing, sections were subjected to immunohistochemistry or stored in 8.5% sucrose until use.

Antibodies

ECCD-2, a rat anti-mouse monoclonal antibody which recognizes mouse E-CD (Shirayoshi *et al.*, 1986), and PCD-1, a rat anti-mouse monoclonal antibody which recognizes mouse P-CD (Nose and Takeichi, 1986) were a generous gift of M. Takeichi (Kyoto University, Japan). Dilutions of monoclonal antibodies were carried out in 150 mmol/L NaCl, 10 mmol/L Hepes, pH 7.4, 10 mmol/L $CaCl_2$ (HMF-Ca buffer), containing 1% (wt/vol) bovine serum albumin (BSA) (Behring, Marburg, Germany).

Immunohistochemistry

Immunostaining was performed by the avidin-biotin-alkaline phosphatase method as previously reported (Navarro *et al.*, 1991; Gamallo *et al.*, 1993; Palacios *et al.*, 1995) with some minor modifications. Frozen sections were first incubated with Tris-buffer containing 2% rabbit serum to block nonspecific antibody binding. They were then incubated with the monoclonal antibodies ECCD-2 and PCD-1, at 1/250 and 1/10 dilutions, respectively, for 1 h at 37°C. After washing in Tris-buffer, pH 7.4, with 1% BSA, tissue sections were incubated with biotinylated rabbit anti-rat immunoglobulins (Dako A/S, Glostrup, Denmark) diluted 1/200 in Tris-buffer, pH 7.4, with 1% BSA for 30 min at 37°C. Tissue sections were then incubated with streptavidin-alkaline phosphatase complex (Dako A/S) for 30 min at 37°C. The alkaline phosphatase activity was developed using 2 mg of naphtol AS-MX-phosphate (Sigma Chemical Co., St. Louis, MO, USA) as substrate dissolved in 200 µL of dimethylformamide (Sigma Chemical Co.), and mixed with 0.1 mol/L Tris-buffer, pH 8.2, made up to 10 mL. To block endogenous alkaline phosphatase, 10 µL of 1 mol/L levamisole (Sigma Chemical Co.) was added. The reaction was completed with 10 mg of fast red dye (Sigma Chemical Co.) as chro-

mogen group. Tissue sections were counterstained with Meyer hematoxylin and mounted for light microscopy study. Negative controls consisted of consecutive sections of each case in which the primary antibody was omitted or replaced with an irrelevant monoclonal antibody of the same species. The mouse skin included in the head sections was used as a positive control.

Acknowledgments

We thank Ms. Inmaculada Briones and Ms. Petra Rubio for technical assistance, and the Fondo de Investigaciones Sanitarias de la Seguridad Social (FIS 92/0505 and 95/0849) for financial support. A. Pizarro was recipient of a grant (BAE FIS 94/502) from the Fondo de Investigaciones Sanitarias de la Seguridad Social.

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Accepted for publication: June 1995