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Conversion of columnar to stratified squamous epithelium in the developing mouse oesophagus

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Abstract

The mouse embryonic oesophagus is initially lined with a simple columnar epithelial layer which changes during the course of development to a stratified squamous tissue. To study the mechanism of this transition, we developed an in vitro model, based on oesophageal explants isolated from E11.5d mouse embryos, which fully recapitulates the normal in vivo development. In this system, the columnar epithelial markers cytokeratins 8 and 18 (K8, 18) were strongly expressed at the beginning of the culture period and decreased in the basal layer of the epithelium at around 5 days of culture. Expression of K8 + 18 persisted in the suprabasal layers of the stratified epithelium for several more days. In contrast, the stratified squamous epithelial marker cytokeratin 14 (K14) was absent at the beginning, and cells expressing it progressively appeared within the basal layer from day 5 to day 9 of culture. The two possible mechanisms for the change are (1) a direct conversion of columnar cells to the basal layer cells of the squamous epithelium; (2) an overgrowth of columnar by squamous cells. Our results show that the first mechanism is operative. Firstly, co-staining for K8 and K14 demonstrates that some cells express both markers during the transition period. Secondly, after electroporation of a construct containing the K14 promoter driving nuclear GFP into the epithelium of E15.5 oesophagus, some cells expressed both K8 and GFP. Thirdly, there is no preferential loss of the columnar cells by apoptosis. Fourthly, inhibitors of apoptosis do not affect the process. Finally, inhibitors of cell division do not affect the process. In terms of the molecular mechanism, inhibitor studies suggest that de novo DNA methylation is required for the loss of the K8 expression but not for the acquisition of the K14 expression. The results show that, in normal development, the squamous epithelium arises from the columnar epithelium by a direct conversion process.

Keywords: Oesophagus; Epithelium; Cytokeratin; Transdifferentiation; DNA methylation

Introduction

The oesophagus connects the pharynx to the stomach and functions mainly as a passage to allow masticated food to enter the gut for further digestion. The mammalian adult oesophagus is lined by stratified squamous epithelium, but in the embryo, it is lined by a columnar epithelium, which becomes replaced at some stage by the squamous epithelium. Studies in the mouse showed that the epithelial layers are initially composed of cuboidal ciliated cells which become replaced by squamous cells from about E17. By this stage, ^{3}HTdR labelling is confined to the basal layer. The superficial cells of the squamous epithelium became keratinised after 8 days postnatal (Raymond et al., 1991). In the human embryo at 8 weeks of gestation, the oesophagus is lined by a pseudostratified columnar epithelium (DeNardi and Riddell, 1991). Ciliated cells appear in the middle third and extend rostrally and caudally so by around 10 weeks a single layer of columnar cells populates both ends of the oesophagus. After 5 months, the stratified squamous epithelium initially appears in the middle third of the oesophagus and extends towards the rostral and caudal ends, replacing the ciliated epithelium (Johns, 1952). These studies clearly describe the replacement of one epithelial

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type by another, but the process has not been studied in relation to cell lineage, so it is not known whether the change results from direct transformation of columnar to basal squamous cells (transdifferentiation) or from the overgrowth of one precursor population by another.

Intermediate filaments are cytoskeletal polymers that provide structural support in the cytoplasm and nucleus of higher eukaryotes and function to sustain the cells against mechanical and non-mechanical stresses (Porter and Lane, 2003; Owens and Lane, 2003). Recent observations suggest that they interact with non-structural proteins (e.g. Jnk) which influence cell growth and death (Coulombe and Wong, 2004). Intermediate filaments belong to a large family of structural proteins and are expressed according to the differentiated state of the cell (Lazarides, 1980). Simple columnar epithelia, including the embryonic oesophagus, express cytokeratin 18 (K18), a type I acidic protein that normally heterodimerises with another intermediate filament, the type II cytokeratin 8 (K8), to form the keratin intermediate filament structure (Owens and Lane, 2003). The stratified squamous epithelium can be divided into basal and suprabasal layers. In the later embryonic oesophagus, cytokeratin 14 (K14) and cytokeratin 5 (K5) are expressed in the basal layer, and K1 and K10 are expressed in the suprabasal layers. Some cytokeratins (e.g. K4 and K13) are found in the oesophagus, but not in other epithelia such as the epidermis so can be considered as tissue-specific markers (Moll et al., 1982). In the work presented here, we have used cytokeratin antibodies K8 and 18 as markers for columnar epithelium and K14 as a marker for stratified squamous epithelium.

In general, the conversion of one cellular or tissue phenotype to another is termed *metaplasia* and can include conversions between stem cells as well as direct conversion of differentiated cells (Slack and Tosh, 2001; Tosh and Slack, 2002). *Transdifferentiation* is a subclass of metaplasia and by definition is an irreversible switch of one already differentiated cell to another, resulting in the loss of one phenotype and the gain of another (Eguchi and Kodama, 1993). There are two important experimental criteria that need to be established for a process to be defined as transdifferentiation. First, the phenotype of the cells before and after transdifferentiation should be clearly defined using morphological appearance and molecular and/or biochemical evidence. Second, the cell lineage (ancestor–descendant) relationship between the two cell types needs to be established (Eguchi, 1995). In this study, we have asked whether the replacement of the columnar by the squamous lining of the oesophagus is due to transdifferentiation or whether it arises from the overgrowth of one cell population by another.

As there were no suitable model systems available to study the conversion of columnar to stratified squamous epithelium, we have devised and characterised an in vitro culture system based on explants of the mouse embryonic oesophagus and used it to investigate the cellular basis of the conversion. We have examined the cell lineage, the role of cell death, the role of cell division and the effects of inhibitors of DNA methylation. The results are as follows:

1. at least some of the cells in the basal layer of stratified squamous tissue arise directly from the columnar cells
2. programmed cell death is not responsible for selective loss of the columnar cells
3. cell proliferation is not necessary for the conversion
4. de novo DNA methylation may be involved

Materials and methods

Isolation and culture of embryonic oesophagus

Pregnant animals were killed by cervical dislocation and the uteri dissected into ice-cold sterile phosphate buffer saline A (hereafter referred to as PBSA). E11.5, E13.5, E15.5 or E17.5 embryos were removed from the deciduas, transferred to ice-cold Minimum Essential Medium (MEM) with Hank's salts, 10% FBS and 50 μg/ml gentamycin and the gut (from pharynx to the intestine) was dissected free. The oesophagus was removed from its position rostral to the stomach and was separated from the trachea.

For organ culture, the oesophagus was placed on a coverslip subbed with APTES and coated with fibronectin (Percival and Slack, 1999). Initially, a cloning ring was placed over the fibronectin-coated area in order to ensure that the explant stayed on the substrate. Basal Medium Eagle (BME) medium with Earle's salts, 20% foetal bovine serum (from Life Technologies), 2 mM glutamine (Sigma) and 50 μg/ml gentamycin (Gibco/Invitrogen) was pipetted dropwise into the cloning ring and then into the rest of the dish up to a total of 2.5 ml. Individual explants were then dropped into the centre of the cloning ring. Twenty-four hours later, the cloning ring was removed, the media decanted and fresh medium added. The cultures were grown at 37°C, 95% air/5% CO_2 in a humidified incubator for up to 20 days. The medium was changed every 2 days.

Histology and immunohistochemistry

Oesophageal cultures (1–20 days) were fixed for 5 min in acetone/methanol (1:1 ratio, for immunostaining of cytoskeleton proteins) or 30 min in MEMFA (10% formalin, 01% MOPS, pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, for immunostaining of membrane, cytosolic and nuclear proteins) at room temperature. After fixation, cultures were washed three times in PBSA and stored in PBSA at 4°C for several days.

Cultures were permeabilised by adding 1% Triton X -100 in PBSA for 30 min prior to immunostaining. Antigen retrieval was performed by adding citrate buffer (pH 6) (LAB VISION, Fermont, CA) for 1 h at 37°C. The cultures were washed three times in PBSA. Non-specific binding sites were blocked for at least 1 h in 2% Roche Blocking