

Syndecan-1 expression during postnatal tooth and oral mucosa development in rats aged from two days to six weeks

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Syndecans are a family of heparan sulphate proteoglycans that regulate cell-matrix interactions that influence cell growth, proliferation and morphology. The aim of this study was to observe changes in the expression of Syndecan-1 in the developing epithelium of the rat oral mucosa and in the epithelial cell rests of Malassez in the developing periodontium of normal rat molars, from late crown development through to early eruption. Immunohistochemistry (Syndecan-1 N-18) and histochemistry (Alcec Blue) were used to observe changes in the expression of Syndecan-1 in rats aged two to 42 days. Results indicated that during normal tooth development in the rat, labelling or staining of variable intensity for Syndecan-1 was demonstrated in the stratified oral epithelium above the stratum basale in the rat tongue and palate, and in ameloblasts of the developing molar in rats aged two to 14 days. Histochemical staining of the pre-dentine and dentine layers was consistent in all specimens. Labelling or staining for Syndecan-1 was negative in the rat periodontal ligament, which may suggest that either Syndecan-1 was not expressed during normal molar root development or that continued work is required for identification of a suitable label in rats.

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Introduction

The cellular micro-environment, including the immediate pericellular matrix, is constantly changing and cells must maintain the ability to monitor molecular information from their external environment in order to maintain normal development and function. Syndecans are a family of transmembrane proteoglycans¹ that regulate the cell-matrix interactions with the micro-environment that influence cell growth, differentiation, proliferation, adhesion and shape.² The role of Syndecans during tooth formation in mice has been described.^{3,4} Matrix components such as collagens I, III and V, and fibronectin and tenascin, bind to Syndecans, initiating changes in cell behaviour that are important in mesenchymal cell condensation and differentiation.^{3,4}

The expression of Syndecan-1 during crown formation in mouse molars is understood to be stage-regulated by epithelial-mesenchymal interactions.^{4,5} During crown formation, Syndecan-1 is expressed by embryonic dental epithelium at the bud stage. With continued tooth development, Syndecan-1 is lost from the dental epithelium and is expressed in the dental mesenchyme. Syndecan-1 is then re-expressed by dental epithelium during the bell stage.^{4,5}

Despite extensive studies documenting Syndecan-1 expression during mouse tooth-crown development,^{4–7} there are no studies describing Syndecan-1 expression during normal root development. The aim of the present study was to observe changes in the expression of Syndecan-1 in both the developing epithelium of the rat oral mucosa, and in the epithelial cell rests of Malassez in the developing periodontium of normal rat molars, from late crown development through to root formation, early eruption and apexification.

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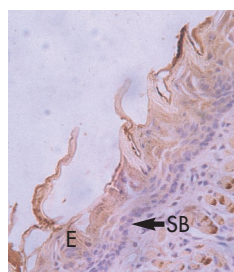


Figure 1a. Syndecan-1 N-18 (1:600) labelling. Photomicrograph of rat tongue mucosa showing positive labelling of the oral epithelium (E) above the stratum basale (SB), and labelling of skeletal muscle. Longitudinal section from a 28-day old rat (original magnification x 200).

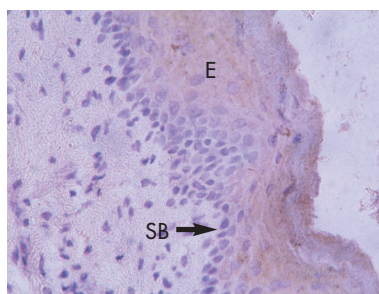


Figure 1b. Syndecan-1 N-18 (1:600) labelling. Photomicrograph of rat palatal mucosa showing positive labelling of the oral epithelium (E) above the stratum basale (SB). Longitudinal section from a 28-day old rat (original magnification x 200).



Figure 1c. Syndecan-1 N-18 (1:600) labelling. Photomicrograph of rat palatal mucosa (M) and the developing rat maxillary first molar (M-1) showing positive labelling of the oral epithelium (E) and ameloblasts (AM). Dentine (D). Longitudinal section from a 14-day old rat (original magnification x 40).

Materials and Methods

A total of 24 Sprague-Dawley rat pups were used and four were killed at each of the following time intervals from birth: 2 days, 7 days, 14 days, 21 days, 28 days and 42 days. The maxillae were dissected and immersion-fixed in 10 per cent neutral buffered formal-saline for 12 hours and decalcified in phosphate buffered 4 per cent EDTA at pH7, before being processed for paraffin embedding. The maxillae were then sectioned either longitudinally or transversely prior to immuno-histochemical labelling (avidin-biotin peroxidase complex technique) or histochemical staining procedures.⁸

Immuno-histochemistry

Following dewaxing and rehydration, 5µm sections were treated with hydrogen peroxide/methanol to quench endogenous peroxidase activity and block any non-specific reaction. After washing in phosphate buffered saline (PBS), standard trypsin and microwave antigen unmasking was performed. Sections were then washed in PBS and incubated with 3 per cent normal horse serum for 30 minutes to block non-specific binding of the secondary antibody. Sections were then incubated overnight with Syndecan-1 N-18 (1:600) goat polyclonal anti-rat/mouse-human antibody.* After washing in PBS, sections were incubated with a biotinyl-

lated rabbit anti-goat immunoglobulin† for 30 minutes and then streptavidin peroxidase‡ for 1 hour, prior to application of diaminobenzidine peroxidase substrate solution.§ Sections were then lightly counterstained with Mayer's haematoxylin prior to mounting. Sections of rat tongue exposed to EDTA served as positive-staining control tissue. Sections of rat tongue exposed to EDTA, from which the primary antibody was omitted, served as a negative-staining control tissue. Control tissues were stained or labelled concurrently with all experimental sections. Positive reference identification of epithelial cell rests of Malassez was provided by labelling adjacent sections with the primary mouse monoclonal anti-rat antibody cytokeratin AE1-AE3 (1: 15,000).^{**}

Histochemistry

Detection of more highly sulphated polyanions such as the heparan sulphate rich Syndecan-1, was achieved in dewaxed, re-hydrated sections stained with Alcec Blue solution (pH 5.8), in 0.42mol/L magnesium chloride†† for 60 minutes.⁸ Increases in counterions (e.g. magnesium chloride) compete for differential binding of available charged groups (e.g. sulphates) on glycosaminoglycans.⁹ Following staining at this critical electrolyte concentration, a pale blue colouration would be permitted.

* Santa Cruz Biotechnology, USA.

† Vector Laboratories, USA.

‡ Pierce, USA.

§ Pierce, USA.

** Cell Marque, USA.

†† Aldrich, USA.

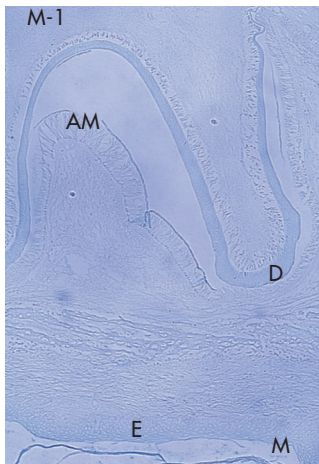


Figure 1d. Alcec Blue (0.42mol/L) staining. Photomicrograph of rat palatal mucosa (M) and the developing rat maxillary first molar (M-1), showing staining of the oral epithelium (E), ameloblasts (AM) and dentine (D). Longitudinal section from a 14-day old rat (original magnification x 40).

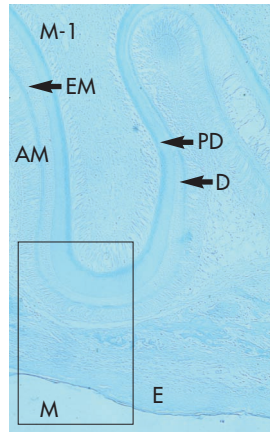


Figure 2a. Alcec Blue (0.42 mol/L) staining. Photomicrograph of rat palatal mucosa (M) and the developing rat maxillary first molar (M-1), showing staining of the oral epithelium (E), and staining of the pre-dentine (PD), dentine (D) and the enamel matrix (EM). Ameloblasts (AM). Longitudinal section from a 7-day old rat (original magnification x 40).

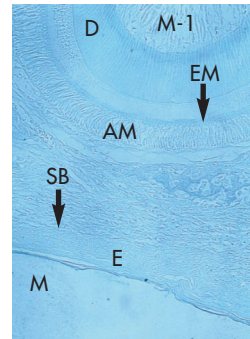


Figure 2b. Alcec Blue (0.42 mol/L) staining. High power photomicrograph of rat palatal mucosa (M) and the rat maxillary first molar (M-1) showing staining of the oral epithelium (E), the enamel matrix (EM), and dentine (D). Stratum basale (SB); ameloblasts (AM). Longitudinal section from a 7-day old rat (original magnification x 200).

Analysis

Sections were examined using conventional light microscopy and assessed for the presence or absence and intensity of Syndecan-1 expression and Alcec Blue staining in the oral epithelium of the rat tongue and palate. The periodontium was examined for epithelial cell rests of Malassez surrounding the mesiobuccal root of the maxillary first molar, from late crown formation through to early eruption. The intensity of Syndecan-1 expression and Alcec Blue staining in the stratified epithelium of the oral mucosa (stratum corneum, stratum granulosum and stratum spinosum) and in the periodontium, was recorded.

The location of staining or labelling of epithelial cell rests of Malassez in transverse sections was made using an eight-point registration system adapted from Andreasen¹⁰ and Andersson *et al.*¹¹ A cross-hair microscope eye-piece was superimposed on the centre of the mesio-buccal root. One line bisected the buccal-lingual root portion and another the mesial-distal root portion, dividing the root and periodontium into four equal quadrants. These quadrants were then subdivided, producing eight segments, for the purpose of localising stained or labelled epithelial rests of Malassez.

In the longitudinal sections, the mesial aspect of the mesio-buccal root was divided into thirds: a cervical, middle and apical third. The furcal

aspect of the root was divided into halves: a furcal and apical half. The location of staining or labelling of epithelial cell rests of Malassez in the periodontium was then recorded within these regions.

Results

Immuno-histochemistry

Immuno-histochemical cytoplasmic labelling of variable intensity demonstrated Syndecan-1 in the stratified epithelial cell layers, stratum corneum, stratum granulosum and stratum spinosum above the stratum basale in the sections of rat tongue epithelium (Figure 1a) and palate (Figure 1b) for all ages examined. Cytoplasmic labelling of variable intensity was observed in the ameloblasts of the developing molar in the sections from 2-day- and 14-day-old rats (Figure 1c). Labelling for Syndecan-1 was negative for the epithelial cell rests of Malassez (Figure 2d) in the developing periodontium for all ages examined, despite positive reference identification labelling (cytokeratin AE1-AE3) of epithelial cell rests of Malassez (Figure 2c) in the adjacent rat tissue sections.

Histochemistry

Histochemical staining (Alcec Blue) for Syndecan-1 was demonstrated by pale blue staining of variable intensity in the stratified epithelial cell layers

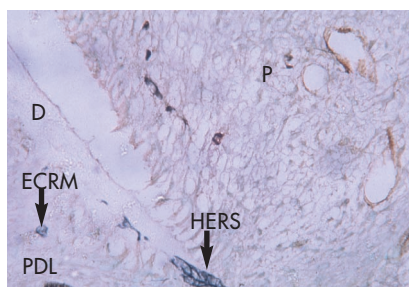


Figure 2c. Cyokeratin AE1-AE3 (1:15,000) labelling. Photomicrograph of the apical periodontal ligament (PDL) third of the first rat maxillary molar (M-1) showing positive labelling of Hertwig's epithelial root sheath (HERS) and epithelial rests of Malassez (ECRM) adjacent to the mesiobuccal root of M-1. Pulp (P); root dentine (D). Longitudinal section from a 28-day old rat (original magnification $\times 200$).

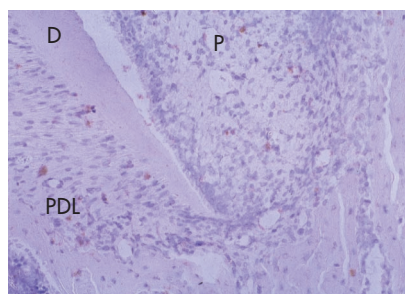


Figure 2d. Syndecan-1 N-18 (1:600) labelling. Photomicrograph of an adjacent tissue section to that shown in Figure 2c, showing negative labelling of epithelial cell rests of Malassez and Hertwig's epithelial root sheath in the apical periodontal ligament (PDL) adjacent to the mesiobuccal root of M-1. Pulp (P); root dentine (D). Longitudinal section from a 28-day old rat (original magnification $\times 200$).

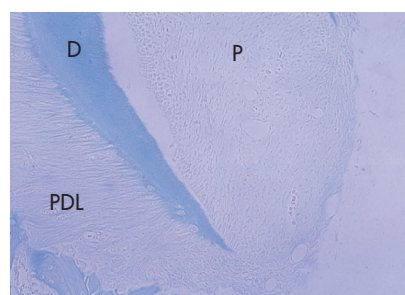


Figure 2e. Alcec Blue (0.42 mol/L) staining. Photomicrograph of an adjacent tissue section to that shown in Figures 2c and d, showing no staining of the epithelial cell rests of Malassez or Hertwig's epithelial root sheath in the apical periodontal ligament (PDL) adjacent to the mesiobuccal root of M-1. Pulp (P); root dentine (D). Longitudinal section from a 28-day old rat (original magnification $\times 200$).

above the stratum basale in sections of rat tongue epithelium and palate (Figures 2a and b) in all ages examined. Alcec Blue staining of the pre-dentine (Figures 2a and b) and dentine (Figures 1d and 2e) layers of the developing molar occurred consistently, with moderate to high intensity in all ages examined. Staining of light intensity was observed in ameloblasts in sections from the 14-day-old rats (Figure 1d). Staining of the enamel matrix occurred in sections from the 7-day-old rats only, and was moderate in intensity (Figures 2a and b). Staining was negative in the epithelial cell rests of Malassez (Figure 2e) of the developing periodontium, despite positive reference identification labelling of epithelial cell rests of Malassez (Figure 2c) in the adjacent rat tissue sections.

Discussion

Oral epithelium-stratum corneum

Syndecan-1 expression and Alcec Blue staining in the stratum corneum of the oral epithelium in the rat tongue and palate was of a higher intensity compared with the stratum granulosum and spinosum. This finding is consistent with Syndecan-1 expression being highest in the stratified epithelium cell-to-cell regions of keratinocytes and lower in the deeper epithelial layers. This result may suggest a role for Syndecan-1 in intracellular adhesion of keratinocytes.¹²

Oral epithelium-stratum granulosum and stratum spinosum

The stratum granulosum and stratum spinosum of the oral epithelium in the rat tongue and palate displayed a reduced staining or labelling intensity for Syndecan-1 when compared with the stratum corneum. This observation may be a consequence of the developmental sequence of cells within the stratified oral epithelium.

Epithelial cells are produced in the basal layer and then undergo proliferation. As cells from the basal layer undergo morphological changes and mature, they form the suprabasal layers (the stratum spinosum and stratum granulosum) and, eventually, the stratum corneum.¹³ In view of this, Bernfield *et al.*² have described how Syndecan-1 expression correlates with the stability and maturity of epithelia, while being reduced in its expression during epithelial proliferation and migration. From the present study, it may be suggested that epithelial cells of the stratum corneum displayed higher intensity staining or labelling having obtained morphological stability following maturation from the deeper epithelial cell layers.

Oral epithelium-stratum basale

The stratum basale of the oral epithelium of the rat tongue and palate did not display labelling (Syndecan-1 N-18) or Alcec Blue staining in the ages examined. Negative labelling or staining of the stratum basale may further support the notion of a reduction in the expression of Syndecan-1 in epithelial cells actively undergoing proliferation and migration.²

Ameloblasts

Staining or labelling of ameloblasts, which develop from the inner enamel epithelium,¹⁴ may have been produced by cytoplasmic components expressing Syndecan-1. This suggestion follows reports describing the intra-cellular distribution of Syndecan-1 within storage and secretory vesicles¹⁵ of various secretory cells, the endoplasmic reticulum¹⁶⁻¹⁸ and, possibly, the cell nucleus.² The staged appearance of ameloblast labelling observed in sections from rats up to 14 days of age may support the notion that Syndecan-1 expression is dependent upon the stability of these epithelially-derived cells.

Epithelial cell rests and the rat periodontal ligament

The absence of Syndecan-1 expression (Syndecan-1 N-18) and Alcec Blue staining in the epithelial cell rests of Malassez of the developing periodontium, for all ages examined, may be interpreted from the developmental fate of embryonic Syndecan-1 as it is modified by epithelial-mesenchymal interactions.² When epithelial cells are induced to change shape, subsequent proteolysis during normal development is an important mechanism. Matrix degradation allows rapid and controlled release of cells from the immediate pericellular matrix, enabling changes in cellular morphology and behaviour.² This has been shown in epithelial-mesenchymal interactions during tooth development where Syndecan-1 expression is reduced in dental epithelium after the early-bud stage, corresponding to a period of rapid epithelial proliferation and differentiation⁴, but increased in expression during the late bell stage.

Based on this model of Syndecan-1 expression during tooth crown development, it may be postulated that the absence of Syndecan-1 labelling or staining observed in the epithelial cell rests of Malassez, may have resulted from a reduction in Syndecan-1 expression following loss of tissue integrity conferred by instability (i.e. shape changes,

proliferation or differentiation) in epithelial cell rests.¹⁹ This mechanism has been suggested by Hayashi *et al.*¹⁷, who describe the Syndecan-1 antigen as being 'lost' or 'masked' when epithelial cells actively proliferate.

Pre-dentine and dentine

Moderate to high intensity staining of Alcec Blue at a critical electrolyte concentration of 0.42 mol/L was consistently demonstrated within the pre-dentine and dentine layers of the developing rat molar for all ages examined. As the proteoglycan content of dentine comprises mainly chondroitin sulphate,²⁰ the results of this study may suggest that heparan sulphate proteoglycans, in particular Syndecan-1, were distributed within the pre-dentine and dentine of the developing rat molar.

A better method for identification of heparan sulphate has been described²¹ in which tissue sections are pre-digested with heparatinase^{† ‡} to remove endogenous heparan sulphate. This generates an unsaturated uronate residue at the terminus of the heparan sulphate stub, which remains attached to the proteoglycan core protein. This unsaturated uronate group is then recognised by the anti-heparan sulphate antibody 3G10.^{§§} Incubation of sections without prior heparatinase treatment displays no labelling with the antibody.

Conclusions

Labelling (Syndecan-1 N-18) or Alcec Blue staining of variable intensity for Syndecan-1 was observed within the stratified epithelium in rat tongue and palate above the stratum basale in rats aged from two to 42 days. Labelling of variable intensity was observed in ameloblasts in sections from rats aged from two to 14 days. Alcec Blue staining of light intensity was observed in ameloblasts of sections from rats aged 14 days. Staining was moderate in intensity in the enamel matrix in sections from rats aged 7 days. Alcec Blue staining of moderate to high intensity was also consistently observed in the pre-dentine and dentine layers of the rat molar at all ages. Labelling or staining for Syndecan-1 was negative in the epithelial cell rests of Malassez in the developing periodontium, which suggests that continued work is required for identification of a suitable label in rats, or that Syndecan-1 was not detected or not expressed, or was reduced in its expression, during the stages of tooth development examined. This finding may be consistent with the notion of proliferating and migrating epithelial cells and suggests the occurrence of these events during the development of Hertwig's

†† Heparin lyase III; ICN, Woburn, MA, USA.

§§ Seikagaku Corporation, USA.

epithelial root sheath and the epithelial cell rests of Malassez in the developing periodontium. Therefore, further research is required in studying older periodontal ligament tissues with the aim of identifying resting (i.e. non-proliferating or non-migrating) epithelial cells.

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