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ABSTRACT

Amelogenin, the major protein component of tooth enamel, is shown to be a cell adhesion protein. Since it had been shown that an amelogenin-containing preparation, Emdogain[®], possessed cell-adhesive activity, we tested the hypothesis that amelogenin was responsible for cell-adhesive activity. Recombinant amelogenin was found to promote adhesion at less than 15 µg/60-mm plate and requires divalent cations for activity. While we found that amelogenin does not bind to collagen or heparin under physiological conditions, it was demonstrated previously that amelogenin does bind to hydroxyapatite. The cell-adhesive activity of amelogenin may play a role in development and may provide a partial explanation for the therapeutic effects of Emdogain[®] in periodontal regeneration.

KEY WORDS: amelogenin, Emdogain[®], cell adhesion proteins, cell adhesion, enamel, ameloblasts, hydroxyapatite.

Amelogenin is a Cell Adhesion Protein

INTRODUCTION

Amelogenin is the most abundant enamel protein, constituting about 90% of the organic matrix of developing teeth (Termine *et al.*, 1980). Immature enamel contains a complex mixture of amelogenin polypeptides, primarily due to the combined effects of alternative RNA splicing and proteolytic processing. In man and cattle, expression from two non-allelic genes on the sex chromosomes generates additional complexity. Amelogenin is mainly expressed by ameloblasts (Hu *et al.*, 2001). Since intact amelogenin cannot be isolated in quantity or readily separated from other amelogenin polypeptides, recombinant amelogenins (rP172 and rM179), which lack the phosphate and N-terminal methionine, are often used for biochemical analysis (Simmer *et al.*, 1994; Ryu *et al.*, 1999).

Emdogain[®] is a heterogeneous mixture of enamel matrix proteins isolated from the crowns of six-month-old pigs. The predominant (> 90%) component of this mixture of hydrophobic proteins is amelogenin. This mixture has been found to promote periodontal ligament cell proliferation and migration (Somerman *et al.*, 1988; Hoang *et al.*, 2000). Animal and human studies suggest that these proteins stimulate periodontal regeneration and influence periodontal ligament cells and bone cells (Boyan *et al.*, 2000; Yukna and Mellonig, 2000).

It is shown here that amelogenin and Emdogain[®] can promote the adhesion of many cell types *via* a divalent cation-dependent mechanism. Many cell adhesion proteins have binding sites for both collagen and heparin. While it is known that amelogenin binds to mineral (Ryu *et al.*, 1998), we show that amelogenin does not bind to collagen or heparin under physiological conditions. The cell-adhesive activity of amelogenin may play a role in the adhesion of ameloblasts to hydroxyapatite or other cell types during development and may provide an explanation for some of the clinical effects of Emdogain[®].

MATERIALS & METHODS

Cell Culture

Cells were maintained in Dulbecco's MEM containing 10% newborn calf serum. The cell lines examined are noted in the text.

Cell Adhesion Assay

Cell adhesion assays were carried out as previously described (Klebe, 1974). In brief, bacterial plastic Petri dishes (Falcon 1007, BD Bio Sciences, Bedford, MA, USA) were coated with amelogenin or Emdogain[®] in minimal attachment medium (MAM) (Klebe, 1974) for 1 hr, and then plates were blocked for 30 min with 0.1% bovine serum albumin (Cat. #2930, EM Science, Gibbstown, NJ, USA) which had been heat-inactivated at 80°C for 3 min. In attachment assays, 2 x 10⁵ cells were added to treated plates containing 5 mL of MAM. After 1.5 hr, plates were washed 3 times with saline to remove unattached cells, and attached cells were trypsinized and counted with an electronic cell counter.

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A supplemental appendix to this article is published electronically only at <http://www.iadr.com>.

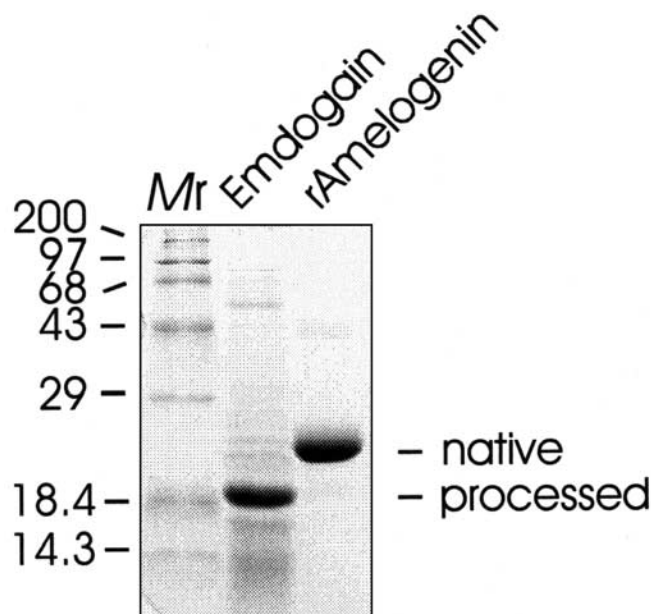


Figure 1. SDS-PAGE analysis of Emdogain® and recombinant porcine amelogenin. Samples of Emdogain® and recombinant porcine amelogenin were analyzed by SDS-PAGE on 15% (w/v) cross-linked polyacrylamide mini-slab gels under reducing conditions and stained with Coomassie Brilliant Blue R-250. The analysis showed that Emdogain® contains several protein species. The molecular masses were clearly detectable from less than 14.3 to over 77 kDa. A major protein band, corresponding to processed ~ 20-kDa amelogenin, was clearly visible in the Emdogain® preparations. In comparison, the native, unprocessed recombinant porcine amelogenin migrated as a single band with a mass of ~ 25 kDa. Mr, molecular-weight markers $\times 10^{-3}$, are indicated.

Preparation of Recombinant Porcine Amelogenin

Recombinant porcine amelogenin (rP172) was expressed from the pET11 expression vector in *E. coli* BL21(DE3) cells (Stratagene, La Jolla, CA, USA) and purified from *E. coli* extracts by selective precipitation in ammonium sulfate (20% saturation), followed by ion exchange chromatography, followed by separation on a C4 reversed-phase column, as described previously (Ryu *et al.*, 1999). While Emdogain® contains many protein species, the predominant species present in Emdogain® corresponds to the processed form of amelogenin (Fukae, 1999) (Fig. 1).

Interactions of Emdogain® and Amelogenin with Heparin and Type I Collagen

Potential interactions of Emdogain® and amelogenin with the extracellular matrix components heparin and type I collagen were investigated by affinity chromatography according to previously described methods (Steffensen *et al.*, 1995) (see Web Appendix, www.dentalresearch.org).

RESULTS

In initial studies, we confirmed the observation of Lyngstadaas (Lyngstadaas *et al.*, 2001) that Emdogain®-treated substrata promoted the adhesion of karyotypically normal human periodontal ligament cells to plastic substrata. Subsequent studies demonstrated that recombinant amelogenin promoted the adhesion of numerous cell lines.

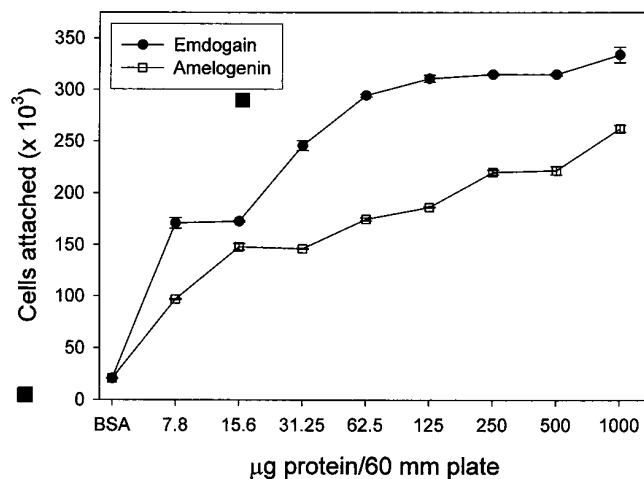


Figure 2. Amelogenin and Emdogain® promote cell adhesion. Plates were coated with the indicated amounts of amelogenin or Emdogain®. Cell adhesion assays were carried out as described in the text, with MG63 cells, and results are presented as the means and standard deviations of 3 determinations. Amelogenin and Emdogain® have similar dose-response characteristics in their ability to support MG63 cell attachment. The concentrations of amelogenin and Emdogain® stock solutions were checked by the BCA assay (Pierce Chemical Co., Rockford, IL, USA), which indicated that the basis of the small difference between amelogenin and Emdogain® dose-response curves was not due to the amount of protein added.

Since normal human cell strains undergo senescence and can vary in their properties from passage to passage, MG63 human osteosarcoma cells, a cell line with well-characterized adhesive characteristics, was chosen for the following quantitative studies.

Cell Adhesion Assays

Cell adhesion assays demonstrated similar dose-response curves for Emdogain® or recombinant amelogenin (Fig. 2). Microscopic examination indicated that both amelogenin and Emdogain® were capable of promoting cell spreading; however, it should be noted that amelogenin did not promote cell spreading as extensive as that promoted by Emdogain® and fibronectin, unless the assay period was extended beyond 1.5 hr (Fig. 3). While cells will attach to, but not spread on, substrata coated with an RGD-containing fibronectin peptide, it is known that a heparin-binding domain of fibronectin or platelet factor 4 is required to promote cell spreading (Woods *et al.*, 2000). It is important to note that amelogenin and Emdogain® can support both cell attachment and spreading.

Many cell adhesion proteins utilize integrins as their cell-surface receptors. Since several integrins are blocked by RGD peptides, we assayed the RGD-containing peptide, RGDS (Sigma cat. #A-9041), for its ability to block MG63 attachment to both amelogenin and Emdogain®. At concentrations as high as 1 mg/mL, which will inhibit $\alpha 5\beta 1$ integrin-mediated cell adhesion (Ruoslahti, 1988), the RGD peptide did not block

amelogenin-mediated cell adhesion (data not shown). Inspection of the human amelogenin sequence did not reveal the presence of an RGD or any other peptide sequence known to bind integrins (Yamada, 1991), and thus, amelogenin may promote cell attachment by a mechanism which does not involve RGD-responsive integrins.

Requirement for Divalent Cations

Many cell adhesion proteins require divalent cations to promote cell adhesion. We found that either Ca^{2+} or Mg^{2+} was required for amelogenin-mediated cell adhesion (Fig. 4). Fibronectin and other cell adhesion proteins also respond to either Ca^{2+} or Mg^{2+} (Klebe *et al.*, 1977).

Cell Type Specificity

While the cell-adhesive activity of amelogenin and Emdogain[®] was initially detected with human periodontal ligament cells (Lyngstadaas *et al.*, 2001), we also found that many other cell lines adhered to both amelogenin and Emdogain[®]. As demonstrated in the Web Appendix (www.dentalresearch.org), we found that recombinant amelogenin promoted the adhesion of several cell lines and promoted adhesion at levels comparable with those to fibronectin. It is well-known that many cell types respond adhesively to fibronectin, vitronectin, laminin, etc., and, thus, it is not surprising that amelogenin also has broad cell-type specificity.

Amelogenin Carbohydrate Binding Activity

Since amelogenin has been shown to have a lectin-like activity that is inhibited by N-acetylglucosamine (Ravindranath *et al.*, 1999), we determined whether N-acetylglucosamine could block periodontal ligament cell adhesion to amelogenin. No inhibition of cell attachment to amelogenin was noted at concentrations of N-acetylglucosamine as high as 100 mM (data not shown), which is reported to inhibit the lectin-like activity of amelogenin completely (Ravindranath *et al.*, 1999).

Absence of Specific, Physiologically Important Interactions of Emdogain[®] and Amelogenin with Heparin and Type I Collagen

It is known that many cell adhesion proteins contain domains with binding sites for both collagen(s) and heparin-like molecules. Thus, we determined whether amelogenin would bind to either gelatin or heparin by affinity chromatographic approaches. Under physiologically relevant conditions, affinity chromatography experiments showed little or no binding of recombinant amelogenin or Emdogain[®] to gelatin or heparin (see Web Appendix, www.dentalresearch.org).

DISCUSSION

There are four classes of cell adhesion proteins, namely, the SAMs, CAMs, cadherins, and selectins. The SAM (surface-adherent material) class of cell adhesion proteins consists of extracellular matrix proteins that bind to membrane-intercalated receptors (integrins) and require divalent cations for activity. Amelogenin has several characteristics of the SAM class of cell adhesion proteins, in that amelogenin is an extracellular matrix molecule, is not membrane-intercalated, and requires divalent cations for activity. Since amelogenin does not contain an RGD sequence or several other sequences known to bind to integrins (Yamada, 1991), the cell-surface receptor for amelogenin may not be an integrin. While the

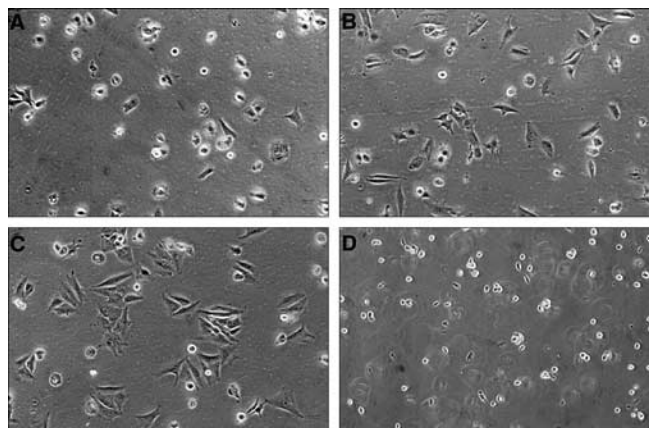


Figure 3. Amelogenin promotes both cell attachment and cell spreading. Using the cell adhesion assay described in MATERIALS & METHODS, we found MG63 cells to attach to and spread on plastic substrata coated with 100 $\mu\text{g}/\text{plate}$ Emdogain[®] (Panel A), 100 $\mu\text{g}/\text{plate}$ amelogenin (Panel B), 100 $\mu\text{g}/\text{plate}$ fibronectin (Panel C), and control (heat-inactivated BSA only) (Panel D). Note that the rounded cells in Panels A-C were attached well enough to resist washing of the plate, while the rounded cells in the control (Panel D)

SAMs often have a binding site for collagen, amelogenin apparently does not (see Web Appendix, www.dentalresearch.org). In contrast, amelogenin does bind to hydroxyapatite (Ryu *et al.*, 1998). Amelogenin not only supports cell adhesion (Fig. 2) but also promotes cell spreading (Fig. 3). It is important to note that the RGD-containing peptide of fibronectin can support cell attachment but requires a heparin-binding domain of fibronectin to mediate cell-spreading (Woods *et al.*, 2000). Based on lack of binding to a heparin-Sepharose affinity column (see Web Appendix, www.dentalresearch.org), amelogenin apparently does not interact with heparin under physiological conditions. Thus, amelogenin has both similarities to and marked differences from members of the SAM class of cell adhesion proteins.

Since amelogenin binds to hydroxyapatite and is present in the organic matrix of developing teeth (Ryu *et al.*, 1999), amelogenin may mediate the adhesion of ameloblasts and other cell types to the mineral component of developing teeth. Since Emdogain[®] was somewhat more active than recombinant amelogenin (Fig. 2), it is possible that one of the processed forms of amelogenin maybe more active than native

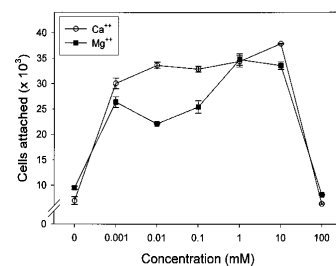


Figure 4. Requirement for divalent cations. Using minimal attachment medium lacking divalent cations, we studied the effect of Ca^{2+} (filled circles) or Mg^{2+} (squares) on amelogenin-mediated cell attachment. The results presented are the means and standard deviations of 3 determinations. Both Ca^{2+} and Mg^{2+} promoted cell attachment in a dose-response fashion at concentrations up to 10 mM, beyond which cell attachment decreased. This result is similar to findings for the divalent cation dependence of fibronectin-mediated cell attachment (Klebe *et al.*, 1977).

amelogenin. The clinical finding that Emdogain[®] promotes periodontal regeneration (Boyan *et al.*, 2000) may indicate that amelogenin has other biological functions in addition to its role in cell adhesion and mineralization.

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