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Short Communication

Immunolocalisation of laminin-1 in keratocystic odontogenic tumors

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Summary

Keratocystic odontogenic tumors (KOTs) are distinct odontogenic lesions frequently affecting the jawbones. They may be associated with nevoid basal cell carcinoma syndrome (NBCCS), and may exhibit disorders involving the extracellular matrix. The aim of this study was to investigate the immunolocalisation of laminin-1 in 20 cases of KOTs in order to contribute to the characterization of this protein, which is little studied in odontogenic tumors. Our results showed laminin-1 in all 20 KOTs studied; its labelling intensity was weak in three cases (15%), moderate in five (25%) and strong in 12 cases (60%). Laminin-1 immunolocalisation was predominantly continuous in 18 (90%) KOTs, including areas of acanthosis, subepithelial split and epithelial buds. Weak immunolabelling was observed in regions exhibiting an inflammatory process, especially in the case of intense inflammation. These findings suggest that laminin-1 does not participate in biological processes such as cystic epithelium-cystic wall separation or the formation of epithelial islands in KOTs. Furthermore, the discontinuous and weak labelling of this protein in the basement membrane of these tumors is probably a consequence of the inflammatory process in the tumor stroma.

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Introduction

Keratocystic odontogenic tumors (KOTs) are locally aggressive tumors affecting the jawbones, especially the posterior region of the mandible, in young adults. The recurrence rate is high and KOTs are often associated with nevoid basal cell carcinoma syndrome (NBCCS) (Shear, 2002; Agaram et al., 2004; Saraçoglu et al., 2005). Histologically, KOTs are characterized by an atrophic parakeratinized stratified squamous epithelium composed of 5–8 cell layers, exhibiting a prominent basal cell layer and surface corrugation (Shear, 2002; Philipsen, 2005). Another common histopathological finding in KOTs is represented by a subepithelial splits of the cystic lining epithelium (Oda et al., 2000; Philipsen, 2005; Kolár et al., 2006), resulting in disorders involving the extracellular matrix, especially the basement membrane.

The extracellular matrix consists of a dynamic and inter-related component of macromolecules that participate in different biological functions such as cell adhesion, growth, proliferation, apoptosis, migration and cell differentiation, with important roles in physiological and neoplastic processes (Kleinman et al., 1981; Terranova et al., 1982; Ekblom et al., 2003; Tzu and Marinkovich, 2008). The basement membrane is a specialized type of extracellular matrix secreted by epithelial and mesenchymal cells, which forms a network consisting of laminin, non-fibrillar collagen, proteoglycans, heparan sulfate and other glycoproteins. In addition, collagen IV and laminin are the most abundant components of the basement membrane of mammals (Ekblom et al., 2003).

Laminins are extracellular glycoproteins composed of a combination of α, β and γ chains, with 16 subtypes being described so far in mammals (Tzu and Marinkovich, 2008). Among these glycoproteins, laminin 1 ($\alpha1\beta1\gamma1$) plays an important role in the embryogenesis of human tissues and is one of the main components of the basement membrane in some adult tissues, participating in the maintenance of cell adhesion and cell survival (Ekblom et al., 2003).

Little is known about the role of extracellular matrix components in the behavior of KOTs. Thus, the objective of the present study was to evaluate the immunolocalisation of laminin-1 in KOTs in order to contribute to the characterization of this protein in these odontogenic tumors.

Material and methods

After approval by the institutional Ethics Committee, 20 cases of KOTs were selected from the archives of the Surgical Pathology Service, School of Dentistry, Federal University of Bahia (FOUFBA), Brazil. Selection was based on the pathological reports and reviewed according to the histological criteria established by Philipsen (2005), as well as on the presence or absence of inflammation. Intensity of inflammation (discrete, moderate and severe) and inflammatory type (mixed, predominantly mononuclear or polymorphonuclear) were also considered.

For morphological analysis, material fixed in formalin and embedded in paraffin wax was cut into 4-μm thick sections. Hematoxylin and eosin-stained slides of each case were submitted to a new histological examination by light microscopy in order to assess the morphological aspects which are characteristics for KOTs (Philipsen, 2005).

Immunohistochemistry was performed on paraffin wax-embedded sections. The tissue sections were deparaffined and rehydrated by routine methods. For antigen retrieval, sections were subjected to enzymatic digestion with 0.4% pepsin (Sigma- Aldrich, Saint Louis, MO) in phosphate-buffered saline (PBS) for 30 min, pH 4.0, and dry heat at 96 °C. The sections were treated with peroxidase block solution (EnVision™ System, DAKO Corporation, Carpinteria, USA) for 10 min to quench endogenous peroxidase activity. Polyclonal antibody against laminin (clone L9393, Sigma, Saint Louis, USA) at a 1:50 dilution was applied for 40 min at room temperature, using an antibody diluent with background reducing components (DAKO Corporation, Carpinteria, USA). Afterwards, a peroxidase-labelled polymer comprising a pool of immunoglobulins anti-rabbit, anti-goat and anti-mouse (Dual Link Polymer, EnVision™ System, DAKO Corporation, Carpinteria, USA), prepared according to manufacturer's instructions, was applied for 30 min at room temperature.

Immunohistochemical reactions were developed with 3,3-diaminobenzidine (EnVision™ System, DAKO Corporation, Carpinteria, USA) prepared according to manufacturer's instructions, for 3 min, as the chromogenic peroxidase substrate and the slides were counterstained with Harris' hematoxylin. Basement membrane surrounding blood vessels of the KOTs themselves served as a positive control for the reactions. Negative controls included replacement of the primary antibody with 1% non-immune bovine serum albumin.

The pattern of immunohistochemical labelling of laminin between primary and recurrent tumors, and non-syndromic ones and those associated with NBCCS was analyzed together by two observers, adopting the criteria described by Poomsawat et al. (2006): continuity of laminin labelling (predominantly

continuous or discontinuous); intensity of immunolabelling (weak, moderate, strong) and relationship between immunolabelling and the inflammatory process were assessed.

The intensity of immunolabelling between groups was compared statistically using a Mann–Whitney test. All statistical calculations were performed with BIOESTAT 5.0 program (Sociedade Civil Mamirauá, MCT-CNPq, Conservation International, Brazil, 2004). The level of significance was set at 5%.

Results

Among the 20 KOT cases studied, five of them were from patients with a diagnosis of NBCCS, and 15 were tumors from non-syndromic patients (sporadic tumors). Fourteen tumors from NBCCS patients were classified as primary tumors and 6 were recurrent tumors.

Histopathologically, all tumors exhibited an atrophic epithelium with a corrugated and parakeratinized surface. Epithelial islands in the cystic wall and areas of subepithelial split were observed in 11 (55%) and 17 (85%) of KOTs, respectively. Inflammation was observed in 12 (60%) tumors and was classified as shown in Table 1.

Laminin-1 immunolabelling was observed in all 20 KOTs studied and was weak in three cases (15%), moderate in five (25%) and strong in 12 (60%). Laminin-1 immunolabelling was predominantly continuous in 18 (90%) KOTs, including areas of acanthosis, subepithelial split and epithelial buds. Regarding the presence of inflammation, weak immunolabelling was only observed in regions where an inflammatory process was present, especially in the case of intense inflammation (Figure 1). No difference regarding the intensity of immunolabelling was observed between primary and recurrent tumors or between sporadic KOTs and those associated with NBCCS (Mann–Whitney test, $p = 0.742$ and 0.545, respectively).

Strong immunolabelling was also localised on the basement membrane surrounding vessel walls, which served as positive labelling control.

Table 1. Intensity and inflammatory type of the keratocystic odontogenic tumors.

Inflammation	Discrete	Moderate	Severe
Predominantly acute	0	0	1
Predominantly chronic	5	4	2

Discussion

Laminin is a non-collagen glycoprotein abundantly present in the basement membrane, which participates in the maintenance of the integrity of the epithelial–connective tissue interface, as well as in biological processes such as proliferation, angiogenesis and invasion (Ekblom et al., 2003; Tzu and Marinkovich, 2008). In the present study, the immunolocalisation of laminin in the basement membrane of KOTs was evaluated. According to the manufacturer, the primary antibody used here recognizes laminin-1, but not fibronectin, vitronectin, collagen IV or chondroitin sulfate types A, B and C. All 20 KOTs studied were immunopositive for laminin-1, with strong labelling being observed in 12 cases (60%). Our results agree with those reported by Poomsawat et al. (2006), but are in contrast to those of Oliveira et al. (2002) who observed weak and discontinuous immunopositivity in cases of KOTs, however, using a different primary antisera to the one employed in the present study. These conflicting results may reflect differences in the immunohistochemical method used. In the present study, we used a polymer system which amplifies the immunohistochemical signal when compared to the streptavidin–biotin method used by Oliveira et al. (2002). Other technical factors, such as the time of enzymatic digestion and the pepsin concentration used for antigen retrieval may also influence immunopositivity. Oliveira et al. (2002) performed antigen retrieval using 1% pepsin for 60 min; in contrast we used 0.4% pepsin for 30 min, which seems to be a less aggressive method.

Areas of subepithelial split are a common finding in KOTs (Philipsen, 2005), suggesting the existence of molecular disturbances involving the basement membrane, and we observed this feature in the majority of the tumors studied. Furthermore, although laminin-1 is one of the most abundant components of the basement membrane (Malinda and Kleinman, 1996; Ekblom et al., 2003; Tzu and Marinkovich, 2008), the present results indicate that the characteristic detachment between the parenchyma and stroma of KOTs is not related to a loss of laminin-1. This is in agreement with the results reported by Poomsawat et al. (2006). Another important histomorphological aspect of KOTs is the presence of epithelial islands in the tumor stroma (Stoelinga, 2001; Philipsen, 2005), which may reflect a possible stromal invasion by KOT cells (Agaram et al., 2004). Although laminins are known to be involved in the process of cell migration (Givant-Horwitz et al., 2005), the observation of continuous immunolabelling in 90% of

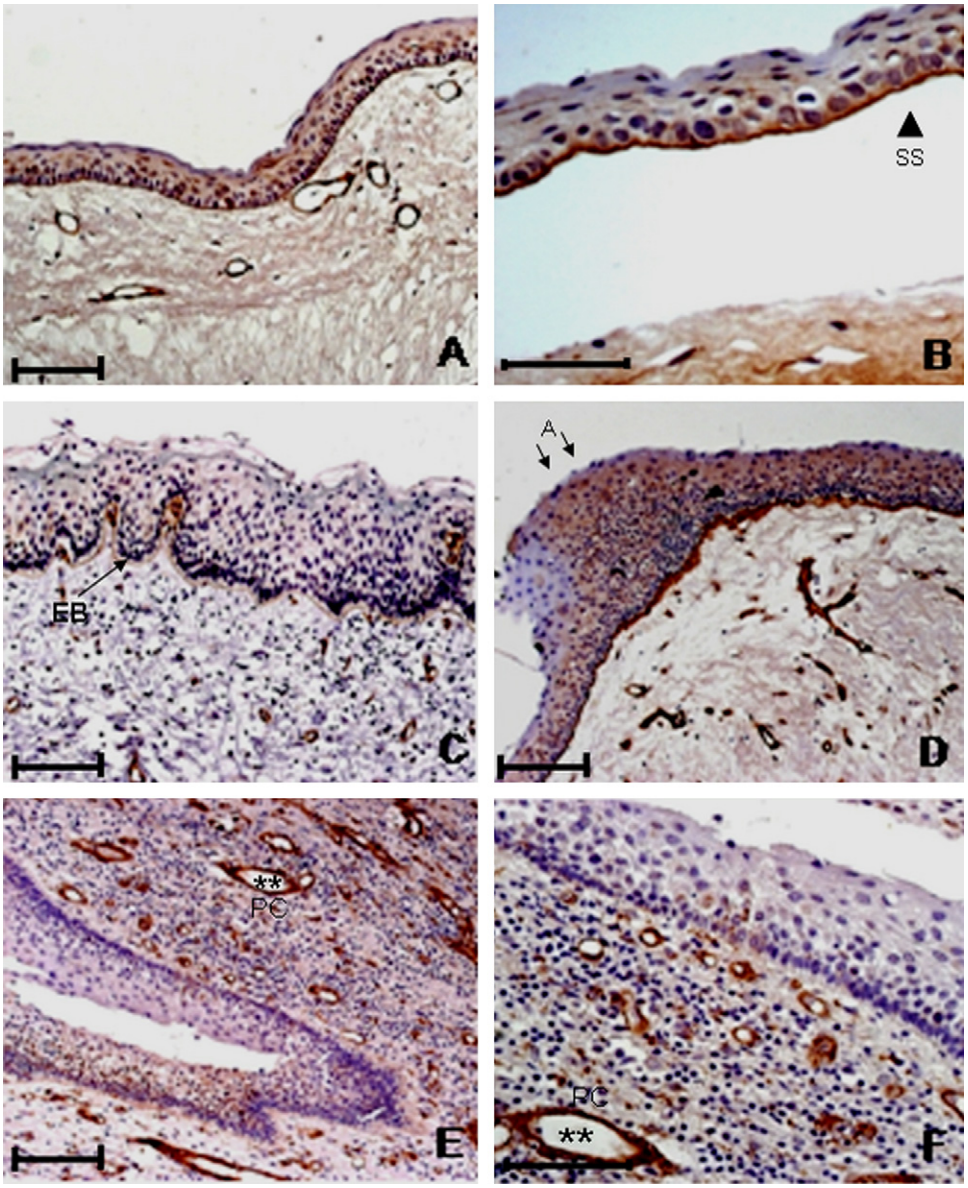

Figure 1. Keratocystic odontogenic tumor exhibiting laminin immunolabelling. (A) Lesion with continuous and strong immunolabelling (scale bar 100 μm); (B) lesion with continuous and strong immunolabelling in area corresponding to the subepithelial split – arrow head (scale bar 50 μm); (C) continuous and weak immunolabelling around epithelial buds – arrow (scale bar 100 μm); (D) continuous and strong immunolabelling in areas showing acanthosis – arrows (scale bar 100 μm); (E and F) weak immunolabelling in areas showing inflammation and basement membrane surrounding blood vessels as positive control – double asterisks (scale bars 100 and 50 μm, respectively).

the KOTs studied rules out the direct participation of laminin-1 in cell migration.

In the present study, no difference in laminin-1 immunolabelling was observed between sporadic KOTs and those associated with NBCCS. Amorim et al. (2004) also found no difference in laminin between these groups. However, in that study most of the cases analyzed were negative for this protein. It should be noted that the immunohistochemical method and type of antigen retrieval used by these authors were the same as those described by Oliveira et al. (2002).

In the present series, an intense inflammatory process (mainly mononuclear) was observed close to the epithelium of KOTs that exhibited discontinuous laminin labelling. Studies investigating chronically inflamed periodontal tissues have demonstrated that the basement membrane responds with the loss of expression and discontinuity of laminin-1 and collagen IV, an event mainly

influenced by enzymes and chemical mediators present in the inflammatory microenvironment (Haapasalmi et al., 1995). We have demonstrated mast cell tryptase in areas with and without inflammation in the KOTs (unpublished data).

Although this study was not able to establish comparisons of angiogenesis among the lesions studied, we found strong immunoreactivity for laminin on the basement membrane surrounding vessel walls that were present in the cystic wall comprising KOTs. Similar immunohistochemical findings were reported by Caffo et al. (2008) assessing secretory meningiomas. It is possible that these results involving angiogenesis influence the growth of KOTs.

Laminin-1 interacts with other components of the basement membrane such as dystroglycan, perlecan and agrin (Malinda and Kleinman, 1996) and presents high affinity for integrins; proteins that are intimately related to the cytoskeleton (Tzu and Marinkovich, 2008). Through this interaction with integrins, laminin-1 participates in cell signaling pathways that are important for the migration and proliferation of neoplastic cells (Ekblom et al., 2003). KOTs present a high proliferative potential as demonstrated in studies investigating proteins such as Ki-67 (Kichi et al., 2005; Gonzalez-Moles et al., 2006; Gurgel et al., 2008), p53 (Gonzalez-Moles et al., 2006; Oliveira et al., 2008; Gurgel et al., 2008) and p63 (Gurgel et al., 2008). In this respect, our results indicate the need for further studies attempting to determine the relationship between laminin-1 and the proliferative potential of KOTs.

Finally, our results suggest that laminin-1 does not participate in biological processes such as epithelium-cystic wall separation or the formation of epithelial islands in KOTs, and that the discontinuous and weak immunopositivity of this protein in the basement membrane of these tumors is probably a consequence of the inflammatory process in the tumor stroma.

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