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ORIGINAL ARTICLE

Survivin expression in odontogenic keratocysts and correlation with cytomegalovirus infection

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OBJECTIVES: The aim of this study was to investigate the expression of survivin, an inhibitor of apoptosis, in odontogenic keratocysts and to compare it to the findings in non-neoplastic jaw cysts – periapical cysts, as well as to establish a possible relationship between survivin expression and human cytomegalovirus presence within these cysts.

MATERIALS AND METHODS: Samples of 10 odontogenic keratocysts (five positive and five negative for the presence of cytomegalovirus, as determined by polymerase chain reaction) and 10 periapical cysts (five positive and five negative for the cytomegalovirus presence) were analysed. The expression of survivin was assessed by immunohistochemical methods, using monoclonal antibody that selectively recognizes the cytoplasmic form of survivin.

RESULTS: All 10 odontogenic keratocysts showed immunostaining for survivin, while all 10 periapical cysts were negative for its presence. There was no correlation between cytomegalovirus presence and expression of survivin within odontogenic keratocysts.

CONCLUSION: Survivin may contribute to the aggressive behavior of odontogenic keratocysts, and thus support the emerging opinion of their neoplastic nature.
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Keywords: survivin expression; odontogenic keratocyst; keratocystic odontogenic tumor; cytomegalovirus; periapical cyst

Introduction

Epithelium-lined cysts of the jawbones are very common in oral pathology. Still, they are unique, as similar lesions are exceedingly rare in the other parts of the skeleton (Neville *et al*, 2002). For a long time, odontogenic keratocysts (OKCs) were recognized as a particularly aggressive kind of odontogenic cysts, featuring destructive growth within the jaws, high recurrence rates and occurrence as a part of the Nevoid Basal Cell Carcinoma Syndrome (NBCCS) (Shear, 2003). A rising body of evidence supports the opinion that OKCs actually are tumors, not cysts (Shear, 2002). Finally, in the new WHO classification they were reclassified as Keratocystic Odontogenic Tumors (KCOTs), defined as benign intraosseous neoplasm of odontogenic origin with characteristic lining of parakeratinized squamous epithelium (Barnes *et al*, 2005).

Despite this, the tumorous nature of OKCs is not completely established. As inhibition of apoptosis is among the most important mechanisms of tumorigenesis, studies regarding expression of apoptosis-related proteins in OKCs may contribute to our understanding of those lesions.

Besides being a well-known inhibitor of apoptosis, survivin is also a promoter of cell proliferation and angiogenesis (Duffy *et al*, 2007). It has been shown that over-expression of survivin in oral squamous cell carcinoma (OSCC) has a prognostic value as it identifies cases with more aggressive phenotype (Lo Muzio *et al*, 2005) and poorer prognosis (Engels *et al*, 2007).

Recent studies suggest that viral infections, in particular herpesviruses, including human cytomegalovirus (CMV) might play a role in the pathogenesis of odontogenic cysts (Slots *et al*, 2003; Andric *et al*, 2007). CMV has a remarkable ability to inhibit apoptosis of infected cells, which is an important step in ensuring viral replication (Andoniou and Degli-Esposti, 2006).

These findings prompted us to study the expression of survivin in OKCs and periapical cysts (PCs), a type of odontogenic cysts that are histologically similar to OKCs, but have a different etiology and pathogenesis (Neville *et al*, 2002). We also tried to establish a possible relationship between survivin expression and the presence of CMV within those cysts.

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Materials and methods

Patients and sample collection

Twenty patients with diagnosed cysts of the jaws enrolled into this study. The study was approved by the Ethical Committee of the School of Dentistry, University of Belgrade. Samples of cystic walls were collected at the time of the surgical removal of the cysts.

Chlorhexidine mouthwashes and high-volume aspiration were used to minimize risk of contamination of samples. Once an appropriate access to the cyst was obtained, part of the cystic wall was excised, placed in an empty plastic vial and immediately frozen until being taken up for the virologic examination. The remaining, larger portion of the cyst was enucleated and used for histopathologic examination and immunohistochemistry.

Criteria for establishing diagnosis of PC were the ability to demonstrate the presence of cystic lumen at the time of surgery, the presence of endodontically treated tooth or tooth with necrotic pulp in connection with the cyst, and histopathologic findings of epithelium-lined cavity with features characteristic of PCs. Diagnosis of OKC was made if typical histopathologic features of OKCs, including parakeratosis of epithelial lining, were present in at least some parts of the cystic wall, and neighboring teeth demonstrated no pulpal or periapical disease.

Cytomegalovirus detection

Viral genome detection has been done as previously described (Andric *et al*, 2007). In brief, samples of cystic wall were processed using a standard proteinase K and phenol-chloroform DNA extraction method. Primers for viral detection were: CMV F:5′-CCACCCGTGGT GCCAGCTCC-3′ and CMV R:5′-CCCGCTCCTCCT GAGCACCC-3′ (Fermentas, Burlington, Canada). The PCR product was 159 bp in size. PCR amplification was performed in a total volume of 25 μl of mixture that contained 3 μl of sample, 1x *Taq* buffer (Promega, Madison, WI, USA), 3 mM $MgCl_2$ (Promega), 0.2 mM deoxynucleoside triphosphates and 0.20 μM of each primer. Initial denaturation of DNA was performed in 94°C for 3 min, followed by 30 cycles of denaturation at 94°C; annealing at 56°C; and elongation at 72°C, 30 s each. Final extension was performed in 72°C for 5 min. Cultured CMV was used as positive and water as negative control in the PCR reactions. The result was considered to be positive if a band of expected size was present on the electrophoresis gel.

Immunohistochemistry

Sections of 5 μm cut from paraffin-embedded tissue were deparaffinized by standard procedures. For antigen unmasking, the sections were heated at 100°C for 20 min in 10 mM sodium citrate buffer at pH 6.0. This was followed by incubation in 3% H_2O_2 for 10 min. Sections were then washed three times in PBS and incubated with a mouse monoclonal anti-human antibody that selectively recognizes survivin (1:50, Abcam, Cambridge, UK), diluted in 10 mM PBS containing 0.3% Triton X-100 and 3% normal goat serum, at room temperature for 1 h. The sections were washed three times in PBS and incubated with a fluorescein isothiocyanate-directly conjugated polyclonal anti-mouse secondary antibody (Abcam, Cambridge, UK), for 1 h at room temperature, diluted to 1:1000 in PBS with 0.25% Triton-X-100 and 1.5% normal goat serum. Samples of pancreatic cancer and transitional cell carcinoma of the bladder were used as positive controls for survivin. Sections without primary antibodies were used as negative control.

Samples were observed with a LSM 510 confocal laser scanning microscope built around an Axioscope 2 FS-mot microscope and using 'Plan-Neofluar' 20× and 40× objectives (Carl Zeiss, Jena, Germany). Confocal images of green fluorescence were collected using the Z-stack mode. Imaging of stained cells was accomplished by using a protocol with an excitation of 488 nm-argon laser light while emission peaks (528 nm) were collected with a BP505–530 filter. Images were processed for display by using LSM 510 Basic software package v. 3.2 (Zeiss).

Results

Samples of odontogenic cysts obtained during surgery have been subjected to CMV detection by PCR in a previous study. For the purposes of immunohistochemical analysis, samples were selected according to the type of cyst and presence or absence of virus in the cystic wall. There were 10 OKCs (five CMV positive and five CMV negative). Also, out of 10 PCs, five were CMV positive and five were CMV negative.

Survivin was clearly expressed in the lining of parakeratinized squamous epithelium of OKCs wall. Cells showing the strongest immunostaining were cells of the basal layer (Figure 1). Survivin displayed a diffuse cytoplasmic staining (Figure 2). In some OKCs, the entire epithelial layer was uniformly stained, while in others, groups of cells demonstrated over-expression of survivin.

The epithelium of the PCs, thicker than the epithelium of OKCs, did not display immunoreactivity to survivin (Figure 3).

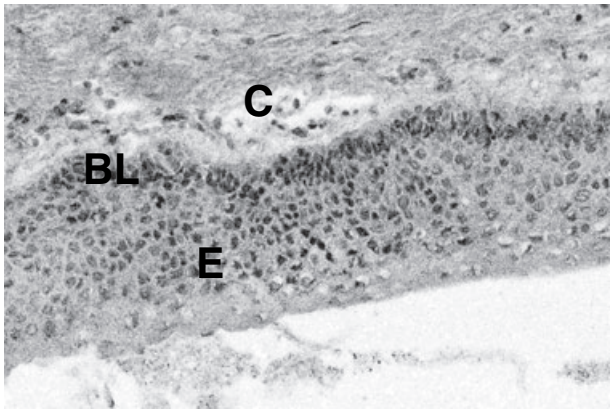

Figure 1 Survivin immunostaining in the epithelium of OKC wall (C-connective tissue, BL-basal layer, E-epithelium); magnification ×20

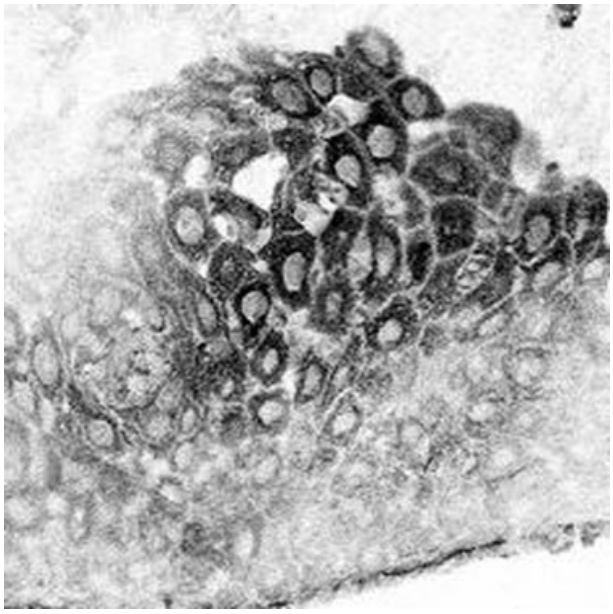

Figure 2 Detail of OKC cytoplasmic immunostaining; magnification ×40

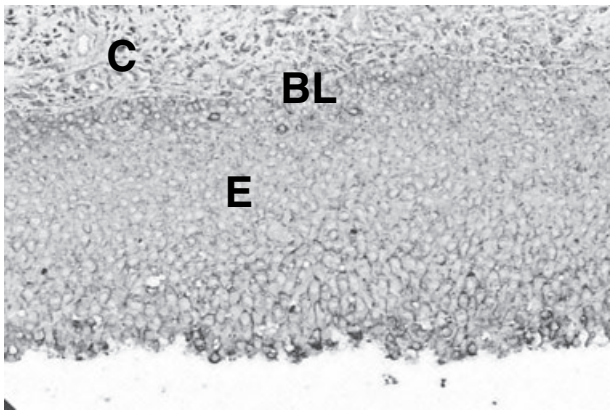

Figure 3 Thick epithelial layer of PC with no evidence of survivin expression (C-connective tissue, BL-basal layer, E-epithelium); magnification ×20

No difference could be noticed in survivin expression between OKCs that were positive to CMV and those that were negative.

Discussion

Several studies have demonstrated that genetic factors are predominant in OKCs etiology (Lench *et al*, 1996; Li *et al*, 2008), and also, that the epithelial lining of these lesions is characterized by increased proliferation rates (Piattelli *et al*, 1998; Thosaporn *et al*, 2004). Cases of solid keratocystic odontogenic tumors have been reported as well (Daley *et al*, 2007).

Though all these studies support the reclassification of OKCs into KCOTs, more evidence is needed to establish their tumorous nature.

The results of our pilot study clearly showed that survivin is over-expressed in epithelial cells of OKCs lining. Moreover, we have detected the cytoplasmic form of survivin, which is considered to be responsible for unfavorable disease outcome in OSCC and for the resistance to therapy in OSCC cell lines (Engels *et al*, 2007). However, effects of nuclear *vs* cytoplasmic expression of survivin are still ambiguous, as some studies have reported that predominantly nuclear survivin over-expression is a negative prognostic factor in oropharyngeal squamous cell carcinomas (Preuss *et al*, 2008). Homogenous and intensive survivin immunostaining of basal and suprabasal cell layers of OKCs stands in contrast to the lack of staining of epithelial cells in PCs. These results indicate that deregulation of cell cycle may be important for the pathogenesis of OKCs. This is in agreement with studies that showed increased cells proliferation rates in OKCs (Li *et al*, 1994, 1995). Finally, the lack of survivin in PCs is in agreement with the view that inflammatory reactions are crucial for their growth (Formigli *et al*, 1995).

It was already shown that the increase in survivin expression might be involved in oncogenesis of odontogenic epithelium (Kumamoto and Ooya, 2004). From that point of view, our results support the opinion that OKCs actually are tumors.

Our results failed to show a relationship between survivin expression and CMV presence. Although CMV is capable of inhibiting apoptosis in infected cells, it seems from our study that such ability is not related to survivin.While discussing the results of this study, quantitative methods should be used for the detection of both CMV and survivin in order to gain more insight into this issue.

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