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Experimental Oral Pathology

Immunohistochemical assessment of extracellular matrix components in syndrome and non-syndrome odontogenic keratocysts

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OBJECTIVE: Investigate the immunohistochemical distribution of fibronectin, tenascin, laminin and collagen IV in syndrome (SOKC) and non-syndrome odontogenic keratocysts (NSOKC).

MATERIALS AND METHODS: Ten cases of SOKC and five of NSOKC were selected and streptoavidin-biotin technique was applied. The specimens were analyzed taking into account the following evaluation parameters: presence, continuity and thickness in the basement membrane and intensity, distribution and association with inflammatory cells in the cyst wall.

RESULTS: Differences could be detected regarding tenascin, fibronectin and collagen IV between the SOKC and NSOKC. Tenascin was present in all cases along the basement membrane in SOKC and in five cases of NSOKC predominated negative areas. Furthermore, tenascin distribution was focal in the cyst wall in SOKC whereas in NSOKC it was diffuse. Concerning fibronectin, it was detected as a discontinuous band when present in SOCK and as a continuous band in NSOKC. Collagen IV was not present in the majority of the cases in SOKC. Negative areas for laminin predominated in the basement membrane in both groups.

CONCLUSIONS: These findings show differences between the immunohistochemical expression of tenascin, fibronectin and collagen IV which might indicate a more aggressive biological behavior of SOKC as compared with NSOKC.

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Keywords: odontogenic cyst; odontogenic keratocyst; extracellular matrix; tenascin; fibronectin; collagen IV; laminin

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Introduction

The odontogenic keratocyst (OKC) represents a unique entity among odontogenic cysts because of its histological features, clinical characteristics and aggressive biological behavior (Ide and Saito, 2003). Based on this, several studies have been carried out in order to establish what drives the distinct features of OKC (Kimi *et al*, 2001; Kubota *et al*, 2002; Lam *et al*, 2002; Shear, 2002; Ide and Saito, 2003). Furthermore, another important aspect of this lesion that should be underlined is that it can represent one component of the nevoid basal cell carcinoma syndrome (NBCS). The NBCS is an autosomal dominant disorder with complete penetrance but variable expressivity characterized primarily by a predisposition to several tumors (Wicking *et al*, 1994; Shafei-Benaissa *et al*, 1998). The most striking component of this disorder is the development of multiple basal cell carcinomas (Lam *et al*, 2002). In addition, this highly penetrant disorder is associated with an array of other features such as OKC, dyskeratotic palmar/plantar pits, skeletal malformations and soft tissue calcifications (Honavar *et al*, 2001; Shear, 2002).

The extracellular matrix (ECM) is a dynamic, intricate network of macromolecules that plays an important role in regulating cellular function during normal and pathological remodeling processes, such as embryonic development, homeostasis, inflammation, tissue repair and tumor development (Thesleff *et al*, 1989; Boudreau and Jones, 1999; Jones and Jones, 2000a; Ioachim *et al*, 2002). Indeed, it has a crucial importance for the maintenance of a correct microenvironment for basic cell functions such as cell adhesion, proliferation and differentiation. The major components of ECM are proteoglycans, collagens, glycoproteins and the structural features of these components provide the basis for their involvement in a variety of interactions that can modulate cell behavior (Chiquet-Ehrismann *et al*, 1995; Ioachim *et al*, 2002).

Tenascin is a high-molecular-weight, multifunctional, ECM glycoprotein, expressed in association with epithelial–mesenchymal interactions (Goepel *et al*, 2000). According to Regezi *et al* (2002), this ECM protein is formed at the epithelial–connective tissue interface and is thought to be produced by both keratinocytes and mesenchymal cells. Fibronectin is a cell matrix glycoprotein, which exists as a number of isoforms and it has been related to support cellular proliferation, migration and early differentiation (Boudreau and Jones, 1999).

Unlike fibronectin and tenascin, collagen IV and laminin are almost exclusively localized in basement membrane as part of specialized structural matrix organization (Boudreau and Jones, 1999; Patarroyo *et al*, 2002). The role of collagen IV in cellular adhesion to the basement membrane and also to other components of ECM has been demonstrated (Abrahamson, 1986; González *et al*, 1994; Tosios *et al*, 1998). Laminin is the most common non-collagenous matrix protein of basement membranes (Patarroyo *et al*, 2002).

In spite of the numerous studies on odontogenic cysts, just a few experiments have focused on the distribution of ECM proteins in these lesions (Lukinmaa *et al*, 1997; Oliveira *et al*, 2002; Silva *et al*, 2002). The aim of the present study is to investigate the immunohistochemical distribution of fibronectin, tenascin, laminin and collagen IV in syndrome (SOKC) and non-syndrome OKC (NSOKC).

Materials and methods

Tissue specimens

Ten NSOKC and five SOKC were selected from the files of the Department of Oral Pathology of the Federal University of Rio Grande do Norte and in all cases the diagnoses were based on the latest World Health Organization (WHO) classification (Kramer *et al*, 1992). All syndrome patients have multiple cysts and all sporadic OKC were single lesions. Moreover, in both groups the cysts were *de novo* lesions. Clinical data are provided in Table 1. Cystic tissues were fixed in 10% buffered formalin and embedded in paraffin. Serial sections of 3 μm thick were taken form the tissue blocks and processed for immunohistochemical examination.

Table 1 Clinical data of the studied groups

	No. of patients	*Sex*	*Age*	*Site*	*Symptoms*
NSOKC	10 Cases	7 ♂	14–28 (range)	9 Mand Post	8 Asymptomatic, 1 swelling, 1 pain
		3 ♀	19.8 (mean)	1 Maxilla Post	
SOKC	5 Cases	3 ♂	11–20 (range)	4 Mand Post	3 Asymptomatic, 1 pain, 1 infection
		2 ♀	15.2 (mean)	1 Maxilla Post	

Mand Post, Mandible Posterior; Maxilla Post, Maxilla Posterior.

Immunohistochemical methods

For immunohistochemistry, the tissue sections were deparaffinized and immersed in methanol with 0.3% hydrogen peroxide to block endogenous peroxidase activity. Immediately afterward, tissue sections were washed in phosphate-buffered saline (PBS). An antigen retrieval, antibody dilution and clone type for tenascin, fibronectin, collagen IV and laminin are shown in Table 2. After treatment with normal serum, the sections were incubated in a moist chamber with the primary antibodies. Following incubation with the primary antibodies, the sections were washed twice in phosphate PBS and treated with streptoavidin-biotin-peroxidase complex method (Dako, Glostrup, Denmark) at room temperature in order to bind the primary antibodies. The peroxidase activity was visualized by immersing tissue sections in diaminobenzidine hydrochloride (D5637; Sigma Chemical, St. Louis, MO, USA), resulting in a brown reaction product. Finally, the sections were counterstained with Mayer's hematoxylin and coverslipped. In our previous experiments, we had found intense staining for fibronectin and tenascin in oral fibrous lesions and tissues sections from these lesions were used as external positive control. As collagen IV and laminin are major components of vessel basal laminae, they were used as an internal positive control. As negative controls, samples were treated as above, except that the primary antibody was replaced by a solution of bovine serum albumin (BSA) in PBS.

Immunohistochemical analysis

The staining pattern of tenascin, fibronectin, collagen IV and laminin were evaluated by using ×40 objective. In each tissue section, all fields were analyzed by two oral pathologists in a double-blind study and the pattern that predominated in the fields was taken into consideration. Tenascin and fibronectin immunoreaction were evaluated in the basement membrane and in the cyst wall whereas collagen IV and laminin were analyzed only in the basement membrane. This was assessed subjectively by at least two oral pathologists. The parameters analyzed in the basement membrane were presence, continuity and thickness staining. In the cyst wall the intensity, distribution and association with inflammatory cells were studied. It was taken into consideration the predominant pattern in each specimen. Thus, a case in which tenascin was recorded as not present in the basement membrane does not mean that the whole area of the membrane was absent but that the absence was the predominant pattern. As this was a descriptive and semi-qualitative study, statistical analysis of the data was not undertaken.

Results

All the specimens were positive for the employed antibodies (Figures 1–7) and the results are listed on Tables 3–5. The blood vessels showed immunoexpression for tenascin, fibronectin, collagen IV and laminin

Table 2 Antibodies and the respective characteristics employed in the immunohistochemical study

Antibody specificity	*Isotype*	*Antibody dilution*	*Incubation period*	*Antigen retrieval*
Tenascin (TN2 clone, M0636, Dako A/S, USA)	IgG 1 kappa	1:100	18 h	0.4% pepsin (37°C) 30 min
Fibronectin (Dako A/S, Denmark)	No isotype	1:500	2 h	1.0% pepsin (37°C) 60 min
Collagen IV (CIV2 clone, M0785, Dako A/S, Denmark)	IgG 1 kappa	1:20	2 h	1.0% pepsin (37°C) 60 min
Laminin (LAM-89 clone, L2871, Sigma, USA)	Mouse IgG1	1:800	2 h	1.0% pepsin (37°C) 60 min

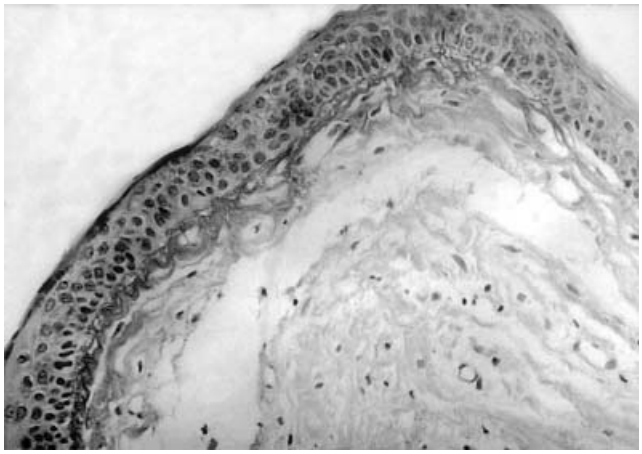

Figure 1 SOKC/Tenascin – continuous immunopositive line in the basement membrane and focal expression in the cyst wall deep to the epithelium. Furthermore, intraepithelial staining is observed (Streptoavidin-biotin peroxidase complex (SABC)-200×)

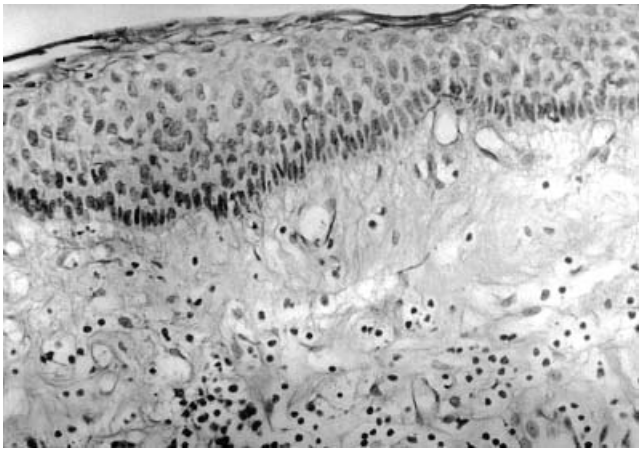

Figure 3 SOKC/Fibronectin – discontinuous pattern in the basement membrane region and diffuse immunoexpression in the cyst wall (SABC-200×)

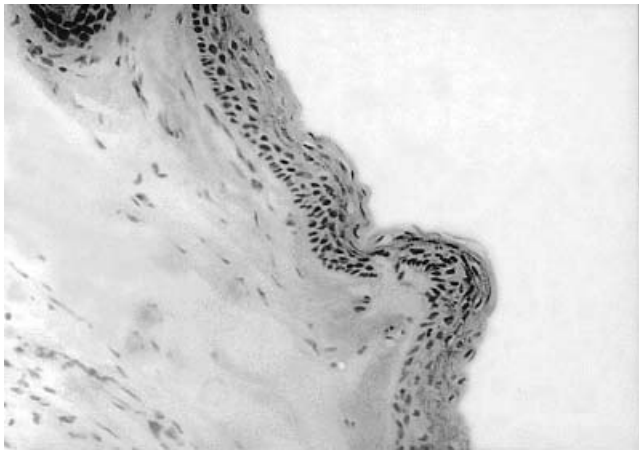

Figure 2 NSOKC/Tenascin – discontinuous immunopositive line in the basement membrane (SABC-200×)

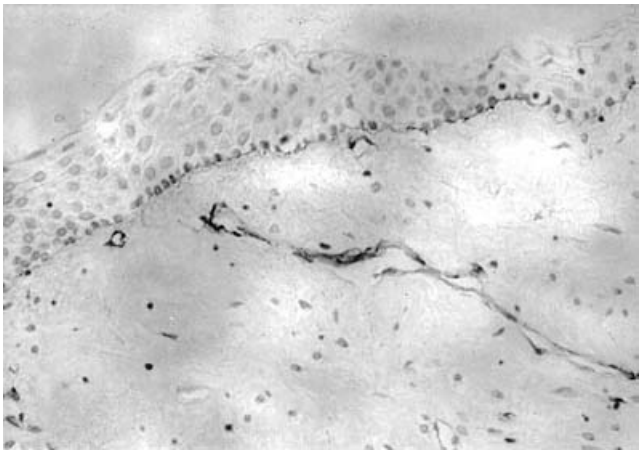

Figure 4 NSOKC/Fibronectin – continuous pattern in the basement membrane region and diffuse immunoexpression in the cyst wall (SABC-200×)

acting as an inherent positive control. Furthermore, some lesions displayed intraepithelial staining for tenascin.

Discussion

The role of matrix proteins in the development and progression of SOKC and NSOKC have not yet been

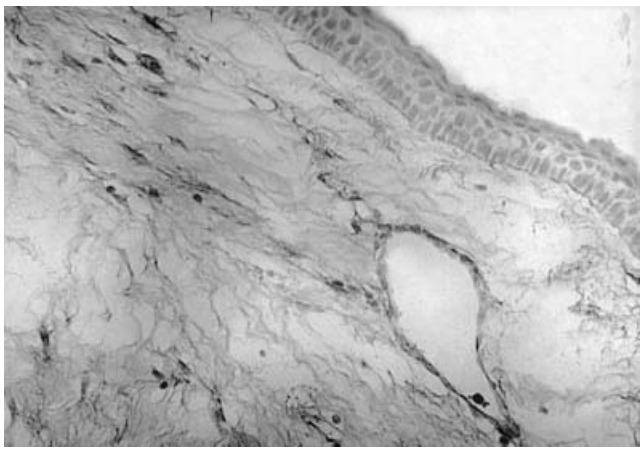

Figure 5 SOKC/Collagen IV – discontinuous immunopositive line in the basement membrane (SABC-200×)

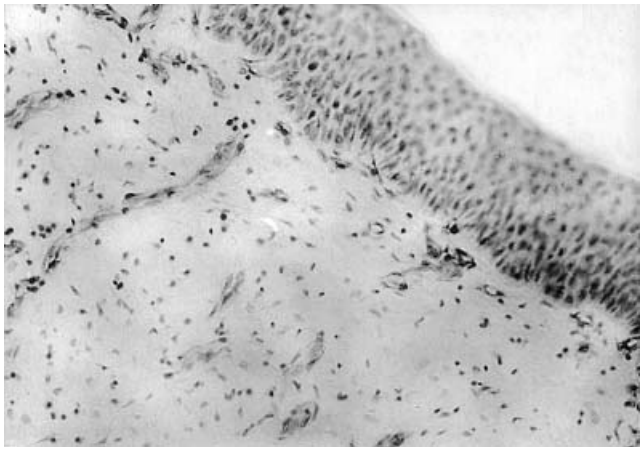

Figure 6 NSOKC/Collagen IV – extensive immunonegative areas in the basement membrane (SABC-200×)

demonstrated. To date, data on the ECM immunoexpression in odontogenic cysts are scarce, and there are only few previous attempts in this regard (Teronen *et al*, 1995; Lukinmaa *et al*, 1997; Hirshberg *et al*, 1999; Silva *et al*, 2002; Wahlgren *et al*, 2003).

Our results showed that tenascin was present in a continuous pattern at the epithelial-connective interface in all cases of SOKC and just 50% of the NSOKC fit this pattern. According to Regezi *et al* (2002), this glycoprotein is overexpressed in processes that require keratinocyte movement and its expression correlates with cell proliferation and migration, such as wound epithelization and connective tissue invasion. To date, several studies have shown that the OKC is well recognized by its invasion potential (Kimi *et al*, 2001; Shear, 2002). Thus, it tends to grow within the medullary cavity of the bone and become a large lesion without causing obvious bone expansion (Li *et al*, 1994). Furthermore, it is stated that SOKCs have a greater capacity to grow and infiltrate compared with the NSOKC (Kimi *et al*, 2001). Taken together, these data indicate that the more significant presence of tenascin at the epithelial-connective interface SOKC can be related

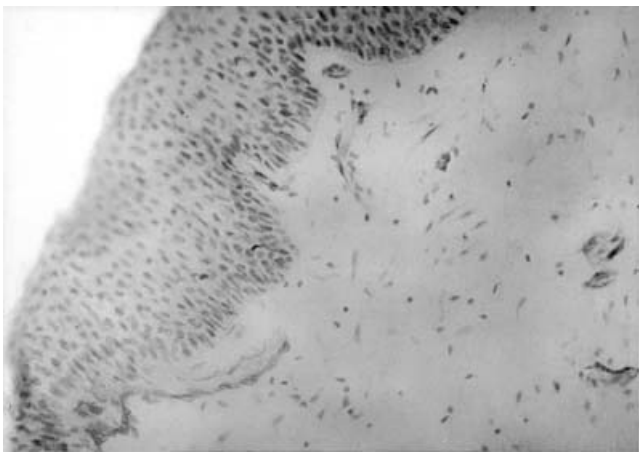

Figure 7 Laminin – predominantly non-reactivity areas in the basement membrane region observed in both groups. Note the immunopositivity in the blood vessels (SABC)

not only to an enhanced ability to infiltrate the contiguous tissues but also to a higher proliferative activity in the epithelial lining. Supporting this statement, Kimi *et al* (2001) performed an immunohistochemical analysis of the cell-cycle and apoptosis-related factors in lining epithelium of NSOKC and SOKC observed differences between the lesions which led to the suggestion that SOKC might be a distinguishable entity from NSOKC.

In regard to the tenascin presence in interstitial matrix, we could not identify substantial differences between the two studied groups but the distribution

Table 3 Thickness of the immunoexpression in the basement membrane in SOKC and NSOKC

	Basement membrane	
	SOKC	*NSOKC*
Tenascin	+ (4 cases) + + (1 case)	– (5 cases) + (2 cases) + + (3 cases)
Fibronectin	– (1 case) + (4 cases)	+ (7 cases) + + (3 cases)
Collagen IV	– (4 cases) + (1 case)	– (3 cases) + (7 cases)
Laminin	– (5 cases)	– (10 cases)

–, absence; +, thin; + +, thick.

Table 4 Intensity of the immunoexpression in the extracellular matrix in SOKC and NSOKC

	Extracellular matrix	
	SOKC	*NSOKC*
Tenascin	+ + (5 cases)	+ (7 cases) + + (3 cases)
Fibronectin	+ (1 case) + + (4 cases)	+ + (10 cases)

+, weak; + +, intense.

Table 5 Immunoexpression pattern of tenascin, fibronectin, collagen IV and laminin in SOKC and NSOKC

	SOKC				*NSOKC*			
	Basement membrane		*Extracellular matrix*		*Basement membrane*		*Extracellular matrix*	
Tenascin	P - 5 cases	C - 5 cases	F - 3 cases	Ai - 1 case	P - 5 cases	C - 3 cases	F - 4 cases	Ai - 2 cases
	A - 0 case	DC - 0 case	D - 2 cases	Nai - 4 cases	A - 5 cases	DC - 2 cases	D - 6 cases	Nai - 8 cases
Fibronectin	P - 4 cases	C - 1 case	F - 0 case	Ai - 0 case	P - 10 cases	C - 8 cases	F - 2 cases	Ai - 1 case
	A - 1 case	DC - 3 cases	D - 5 cases	Nai - 5 cases	A - 0 case	DC - 2 cases	D - 8 cases	Nai - 9 cases
Collagen IV	P - 1 case	C - 0 case	–	–	P - 7 cases	C - 1 case	–	–
	A - 4 cases	DC - 1 case	–	–	A - 3 cases	DC - 6 cases	–	–
Laminin	P - 0 case	C - 0 case	–	–	P - 0 case	C - 0 case	–	–
	A - 5 cases	DC - 0 case	–	–	A - 10 cases	DC - 0 case	–	–

P, present; A, absent; C, continuous; Dc, discontinuous; F, focal; D, diffuse; Ai, associated with inflammation; Nai, non-associated with inflammation.

pattern was frequently focal in SOKC. Interestingly, it a diffuse tenascin distribution was observed in the wall of the NSOKC. Some experiments have demonstrated that tenascin has an important role during tumorigenesis and it has been reported to be expressed in the stroma of many tumors, including gliomas, mammary tumors, non-invasive and squamous cell carcinomas and lung carcinomas (Jones and Jones, 2000a,b;). Recently, some authors have supported the hypothesis that the OKC represents a benign cystic neoplasm (Shear, 2002). According to this hypothesis, a more diffuse tenascin distribution might have been expected in the wall of the SOKC but this was not observed in the present study. However, it is important to emphasize that OKC is considered to have distinct growth sites (Scharffetter *et al*, 1989) and this may be one plausible explanation for the focal distribution observed in the syndrome group.

Fibronectin comprises a family of closely related, dimeric glycoproteins which are present both as soluble plasma constituents and within connective tissues and play an important role in embryonic development by mediating cell adhesion and migration (Boudreau and Jones, 1999; Labat-Robert, 2002). This protein was present as a discontinuous line in the basement membrane in SOKC. This finding may be related to a higher proliferative potential in the cystic structure of SOKC compared with the NSOKC as the discontinuity could facilitate epithelial–mesenchymal signalizing relations.

One interesting finding observed in the present experiment was the weak immunoexpression of fibronectin in the cyst wall of SOKC. According to Labat-Robert (2002), during tumor growth, invasion, metastasis, angiogenesis, ECM proteolysis is a crucial step. Several enzymes can degrade ECM proteins and cell-associated proteins (Werb, 1997). Some studies indicate that the protein fragments of the ECM may show new properties that the native protein does not exhibit (Barlati *et al*, 1983). In regard to the fibronectin, one of the new properties of its fragments could be related to lesion growth as well as invasion. In our study, the weak expression of fibronectin in the SOKC may indicate that this protein could be degraded and, consequently, was not strongly detected by immunohistochemically. Thus, this finding might suggest that the lesions of SOKC have a greater potential to grow and invade. Another point, is the fact that fibronectin and tenascin have either synergic or antagonic relations (Chiquet-Ehrismann *et al*, 1995). The tenascin was strongly observed in the cyst wall of SOKC. We found a weak expression of fibronectin. Thus, an antagonic relation between tenascin and fibronectin was observed in our experiment.

Seven cases of NSOKC showed the presence of collagen IV in the basement membrane and six of them as a discontinuous line. This could be associated with the proliferative potential of this cyst inasmuch as the disruption of the basement membrane might result in alterations of epithelial–mesenchymal relations. In the SOKC, four of the five studied lesions showed extensive negative areas for collagen IV in the basement membrane which suggest a greater degree of epithelial–mesenchymal interactions.

The results of the laminin were the same in both groups. The absence of the laminin at the basement membrane lead us to speculate about the proliferative capacity of the OKC as well as the disruption between the epithelium and the cyst wall which is a striking characteristic of this lesion. The tendency for a greater frequency of OKC recurrence than other types of odontogenic cysts could be the result of epithelial lining disruption, which may be related to a weak expression of laminin. Bellinghieri *et al* (1997) demonstrated a striking role of ECM proteins in the development of renal cysts in patients with renal polycystic disease and concluded that the absence of laminin in renal cysts could be linked to its development and growth which support our finding.

Another interesting finding was the intra-epithelial tenascin expression observed in the epithelium of both SOKC and NSOKC. This finding was also encountered by Lukinmaa *et al* (1997) who found intra-epithelial tenascin immunostaining in calcifying odontogenic cyst. Based on this, it can be speculated that tenascin has a role on the epithelial cavitation process of OKC, but this possibility has to be investigated in future researches.

In conclusion, our results suggest differences between the immunoexpression of tenascin, fibronectin and collagen IV in SOKC and NSOKC. Taken together,

these findings might indicate a more aggressive biological behavior of SOKC as compared with NSOKC.

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