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Morphological and immunocytochemical characterization of snake-like chromatin cells

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Summary. Snake-like chromatin (SLC) is a nuclear alteration occurring under various pathological conditions and in different tissues. The aim of this study was the morphological and immunocytochemical characterization of SLC-positive conjunctival epithelial cells from keratoconjunctivitis sicca (KCS) patients.

Impression cytology specimens from the upper bulbar conjunctiva of 10 controls and 10 KCS patients with a high incidence of SLC cells were assessed, the morphology of SLC nuclei evaluated by light microscopy, and proliferation markers, nucleolar proteins, lamins and cytokeratin filaments detected immunocytochemically.

In KCS patients, SLC cells with a normal nuclear shape, with nuclear membrane notching (2.3% of cells) and with binuclear dumb-bell structures (4.4% of cells) were observed. The most striking features of SLC cells were the absence of an A/C lamin signal, the redistribution of fibrillarin into two spots adjacent to SLC structures and cytokeratin 14 positivity in the strangulation belt of the dumb-bell structures.

The deficiency of lamin A/C is the probable reason for the disintegration of chromatin from the nuclear lamina in SLC cells. The occurrence of SLC-positive cells, SLC-positive dumb-bell shaped nuclei and SLC-positive binucleated cells, together with the absence of mitotic markers, leads to the conclusion that the SLC phenomenon might be a form of nuclear segregation.

Key words: Impression cytology, Keratoconjunctivitis sicca, Snake-like chromatin, Immunocytochemistry

Introduction

Snake-like chromatin is a peculiar arrangement of condensed chromatin located along the longitudinal axis of the cell nucleus. The nucleoplasm in the region between the condensed chromatin and the nuclear envelope is extremely hypochromatic, with an almost empty appearance. Nucleoli are inconspicuous or missing (Murphy and Becker, 1966; Pienaar and Price, 1967; Marner, 1980).

The presence of snake-like chromatin in eye cells was first observed in patients with keratoconjunctivitis sicca, almost exclusively in the epithelium of the upper bulbar conjunctiva (Marner, 1980). The occurrence of this phenomenon was later described in association with contact lens wear (Aragona et al., 1998; Gurdal et al., 2003), ocular surface pathology and eye surgery (Rivas et al., 1992; Tsubota et al., 1992). SLC structures were also observed in different tissues and cell types in congenital and inflammatory heart diseases (Murphy and Becker, 1966; Favara and Moores, 1987) and in epithelial cells of the buccal mucosa in patients with major anaemias or oral lesions (Silverman, 1965; Diversi et al., 1966). The appearance of SLC accompanies several pathological conditions including inflammation, immune imbalance (Buja et al., 1976; Karabulut et al., 1999), neoplasia (Ragsdale, 1973), hypoxia (Diversi et al., 1966; Favara and Moores, 1987) and mechanical irritation (Knop and Reale, 1994). SLC has also been described in healthy normal tissue but at a much lower frequency than that occurring pathologically (Bjerrum, 1998).

The pathological mechanism that triggers SLC formation still remains obscure. Opinions that SLC is a sign of degeneration (Murphy and Becker, 1966) or regeneration (Kobayashi et al., 1992), including cell proliferation (Boor and Ferrans, 1982), can be found in the literature.

SLC cells with nuclear membrane notching and different degrees of dumb-bell shaped structures resembling nuclear division have been observed (Diversi et al., 1966; Meller, 1999). Due to the proposed relationship of SLC to cell division, the presence of proliferation-associated protein Ki-67 and proliferating cell nuclear antigen (PCNA) was assessed in the present study. Because of the contradictory results about the

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presence of nucleoli in SLC cells (Favara and Moores, 1987; Knop and Reale, 1994), as well as because of nucleolus disassembly during cell division (Olson et al., 2002), the nucleolar proteins fibrillarin and B23 (nucleophosmin) were examined. Furthermore, we investigated the occurrence of lamins A/C and B, structural components of the nuclear lamina that have an important role in the anchoring of chromatin to the innermost nuclear membrane (Gerace and Blobel, 1980). To characterize 10 nm wide filaments acting as a strangulating belt around some SLC-positive dumb-bell structures, as were previously observed using electron microscopy (Meller, 1999), cytokeratin antibodies were used.

Materials and methods

Subjects, clinical examination

The study was approved by the Ethics Committee of the University (Charles University, Prague) and followed the tenets set out in the Declaration of Helsinki for research involving human subjects. Group 1 consisted of 10 control healthy volunteers (8 women and 2 men, mean age 46.0±9.8 years), who did not wear contact lenses, had no symptoms of ocular irritation, had normal aqueous tear production as confirmed by a Schirmer test, and had no signs of ocular surface disease. Group 2 consisted of 10 patients suffering from KCS (8 women and 2 men, 46.5±6.1 years), with a high incidence of SLC cells (more than 1000 cells/specimen). KCS was diagnosed according to the Copenhagen criteria, i.e. at least two of the following tests were abnormal: Schirmer test ≤ 10mm/5min, BUT ≤ 10s, rose bengal staining ≥ 4 (van Bijsterveld, 1969; Manthorpe et al., 1986). All 10 patients suffered from both aqueous and lipid tear deficiency. KCS was associated in two of the patients with graft versus host disease, in one with Kawasaki syndrome, in one with ocular cicatricial pemphigoid, in one with rheumatoid arthritis, and in two with primary Sjögren's syndrome. Three individuals had no apparent systemic disease.

Impression cytology

Impression cytology was carried out bilaterally on the upper (at the 11-12 and 12-13 o'clock positions) conjunctiva, 2 mm posterior from the limbus. Strips of nitro acetate cellulose filter papers (GSWP 0.4700, pore size 0.22 μm) were gently pressed by a glass rod for 3 to 5 seconds onto the conjunctival surface. The specimens for morphological characterization were stained with a combination of periodic acid-Schiff (PAS) and Gill's modified Papanicolaou stains (Gill et al., 1974; Tseng, 1985; Martinez et al., 1995). Imprints from the same location taken on Biopore MILLICELL®-CM membranes (PICM 01250, Millipore, Bedford MA) were used for immunocytochemistry. The membranes were stored at -70°C until they were processed.

SLC cell morphology

The cellular morphology was studied by light microscopy at a magnification of 1000x. One thousand epithelial cells were scored in both the control and KCS groups by two masked observers, and the percentages of cells with SLC and those showing nuclear membrane notching, dumb-bell nuclei and binucleate cells were calculated.

Indirect immunofluorescence

The Biopore membranes were released from plastic holders (acetone for 50 seconds) and placed cell side up on round 12 mm coverslips, then were fixed with acetone and permeabilised with 0.2% triton (Triton X 100, Sigma) in phosphate-buffered saline. The specimens were incubated with the following antibodies: mouse monoclonal anti-fibrillarin, a gift from M. Pollard (Pollard et al., 1997), mouse monoclonal anti-Ki-67 (Transduction Laboratories, BD Biosciences, San Diego, CA), mouse monoclonal anti-PCNA (DAKO, Glostrup, Denmark), mouse monoclonal anti-pan cytokeratin and anti-cytokeratin peptide 14 (Sigma-Aldrich Corp, St Luis, MO, USA), rabbit polyclonal anti-B23 (nucleophosmin), mouse monoclonal anti-lamin A/C and goat polyclonal anti-lamin B (all from Santa Cruz Biotechnology, Santa Cruz, USA). FITC conjugated anti-mouse, anti-goat or anti-rabbit IgG or IgM (all from Jackson ImmunoResearch Laboratories, West Grove, USA) were used as secondary antibodies. The specimens were mounted with Vectashield-propidium iodide (Vector Laboratories, Inc. Burlingame, CA). Negative control specimens (primary antibody omitted) were run with each experiment. Slides were examined by fluorescent microscopy using an Olympus BX 51. At least five specimens were used for immunostaining experiments from each experimental group. Two to five hundred cells were examined in detail. The percentage of positive cells was determined for each specimen.

Results

SLC cell morphology

The nuclei of SLC-negative cells from both the control and KCS groups showed a normal chromatin appearance, while SLC cells were characterised by condensed snake- or stick-shaped chromatin (Fig. 1). The low number of SLC cells in control specimens (less then 0.1% of cells, in only two of ten controls) did not permit a valid examination of SLC in the control group.

SLC cells in the group 2 specimens were most abundant in clusters composed of a few to more than 100 cells, located in areas with minimal goblet cells. SLC structures were found mostly in squamous cells with different degrees of keratinization. No nucleoli were observed in SLC cells. SLC structures showed various intermediates between a straight stick and a curved

snake appearance (Fig. 2A,B). Different degrees of chromatin detachment from the nuclear membrane were observed. In some cells, developing SLC structures were still connected by multiple chromatin filaments to the nuclear membrane (Fig. 2C), in others complete detachment was observed. In these cases chromatin fibrils extended for various distances from the SLC structures toward the nuclear membrane (Fig. 2D). Sometimes, only central, fully condensed SLC structures were seen (Fig. 2E), connected to the cell membrane only at the nuclear poles with no chromatin strands. SLC-positive cell structures resembling nuclear segregation were present: different degrees of notching of the SLC nucleus (2.3% of total SLC cells) (Fig. 2F) and binuclear dumb-bell forms in which two nuclei were connected by a chromatin bridge of various length (4.4%) (Fig. 2G,H). Rarely, double binuclear forms were observed (0.1%). Binuclear cells were also observed in 2.6% of the SLC-positive (Fig. 2I) as well as 2.6% of SLC-negative cells in group 2. The occurrence of nuclear membrane notching and dumb-bell structures was also observed in SLC-negative cells but at a much lower frequency (less than 0.2%).

Indirect immunofluorescence

Immunostaining for Ki-67 and PCNA exhibited weak scattered positivity in control cells (means of 23% and 17% of all cells, respectively) as well as in SLC-negative pathological epithelium from group 2 (means of 8% and 1%, respectively). SLC-positive cells were always negative in all examined subjects (Fig. 3A,B). In controls, one to five fibrillarin-positive nucleoli were observed in 84% of cell nuclei, with little difference among the specimens. In group 2, all SLC-negative cells with a fibrillarin signal (66%) showed a characteristic nucleolar pattern. In 42% of SLC-positive cells in group 2, a signal for fibrillarin was not observed. The relocation of the fibrillarin signal location in SLC-positive cells was observed in all cells (58%) with fibrillarin staining. In 88% of these SLC-positive cells, the signal was mostly observed in two spots closely adjacent to the central part of the SLC structure, and in 12% of SLC-positive cells a weak signal was interspersed along the centre of the SLC structure (Fig. 3C). Positive staining for B23 was observed in 43% of control cells. The number of SLC-negative cells with a positive signal decreased to 1% in group 2, and staining completely disappeared in SLC-positive cells in this group (Fig. 3D). Staining for lamin A/C and lamin B displayed a characteristic pattern with strong inner nuclear membrane positivity in 92% and 93% of cells, respectively, in control specimens. In the SLC-negative cells of group 2, the number of cells staining positively for lamin A/C was 89% and for lamin B 90%. In the SLC-positive cells of group 2, a pronounced decrease in the number of cells staining for lamin A/C was observed (17%), but not in the number of cells staining for lamin B (90%), where only a weaker signal intensity was noted (Fig. 3E,F). The filamentous cytoplasmic pattern of the signal as well as the number of pan-cytokeratin-positive cells did not significantly differ between control SLC-negative (95%), KCS SLC-negative (87%) and KCS SLC-positive (82%) cells (Fig. 3G). The signal for cytokeratin 14 was interspersed in the cytoplasmic network of SLC-negative cells of groups 1 and 2 (in 85% and 87% of cells, respectively). The same cytoplasmic network signal pattern was present in 79% of SLC-positive cells. In dumb-bell SLC-positive structures, besides the cytoplasmic signal, positivity was observed in the strangulation belt in 71% of the dumb-bell structures (Fig. 3H).

Discussion

The pathogenesis as well as the mechanisms underlying the formation of SLC are still unclear.

We were interested to determine if some markers of proliferation could be found in cells with this nuclear alteration. The absence of condensed chromosomes and mitotic spindles characteristic of mitosis as well as the

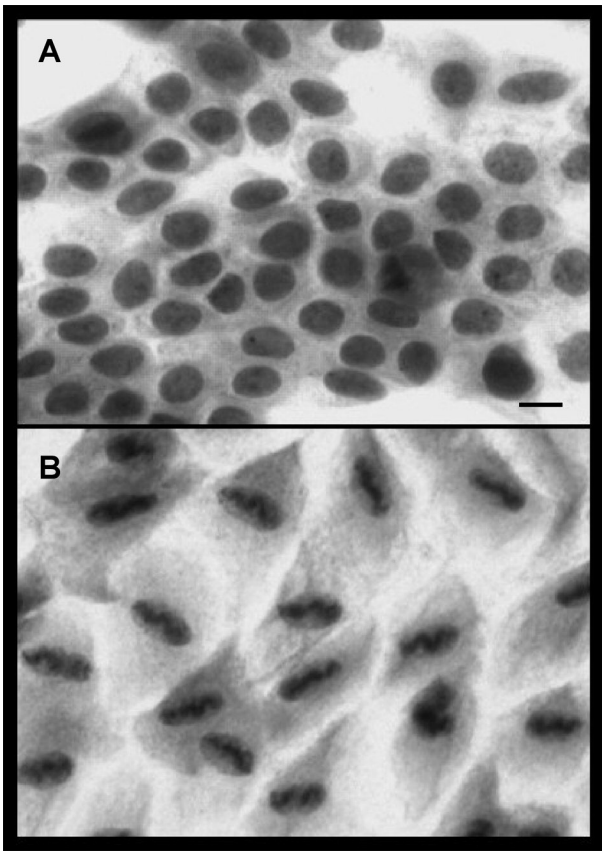

Fig. 1. The difference between the morphology of control epithelial nuclei with a normal chromatin arrangement **(A)** and snake-like chromatin in cells from a conjunctiva of keratoconjunctivitis sicca patient **(B)**. Scale bar: 10 μm.

absence of the proliferation proteins Ki-67 and PCNA in SLC cells does not argue for the proliferative capacity of these terminally differentiated cells.

Surprisingly, we found CK 14, which is characteristically expressed by the basal epithelium (Kasper et al., 1988), to be present in surface epithelial cells. Only basal and suprabasal cells showed CK 14 positivity when immunohistochemical staining was performed on frozen conjunctival sections. The positivity found in the impression cytology specimens may be explained by the unmasking of the CK 14 epitope during the permeabilisation step performed on the Millicel membranes.

From a morphological point of view, the different degrees of chromatin disintegration from the nuclear lamina, the different degrees of condensation of the SLC structures, nuclear membrane notching and strangulation and the formation of binuclear dumb-bell forms connected with chromatin bridges may reflect the dynamics or evolution of snake cells.

Our immunocytochemical results help to elucidate these morphological observations. The release of tensioned chromatin and its subsequent condensation can be caused by the disintegration of A/C lamin. Progressive changes in nuclear architecture, including the loss of peripheral heterochromatin caused by structural alterations in lamin A, have been observed during various "laminopathies" (Chen et al., 2003; Goldman et al., 2004). Our observation of a lack of nucleoli in SLC cells is consistent with earlier findings from light and electron microscopy (Knop and Reale, 1994), although the presence of nucleoli has also been reported (Favara and Moores, 1987). The complete disappearance of B23 in SLC-positive cells and fibrillarin relocation can be related to the different behaviour of the two proteins during nucleolar rebuilding (Olson et al., 2002), which may occur during chromatin segregation and the formation of binuclear structures. The close apposition of fibrillarin to condensed snake-like chromatin may explain the negative results from light microscopy, where nucleoli can be masked by the condensed chromatin mass. The spiral accumulation of cytokeratin 14 around the nuclear surface can participate in the process of nuclear membrane notching and strangulation and the subsequent formation of binuclear dumb-bell forms

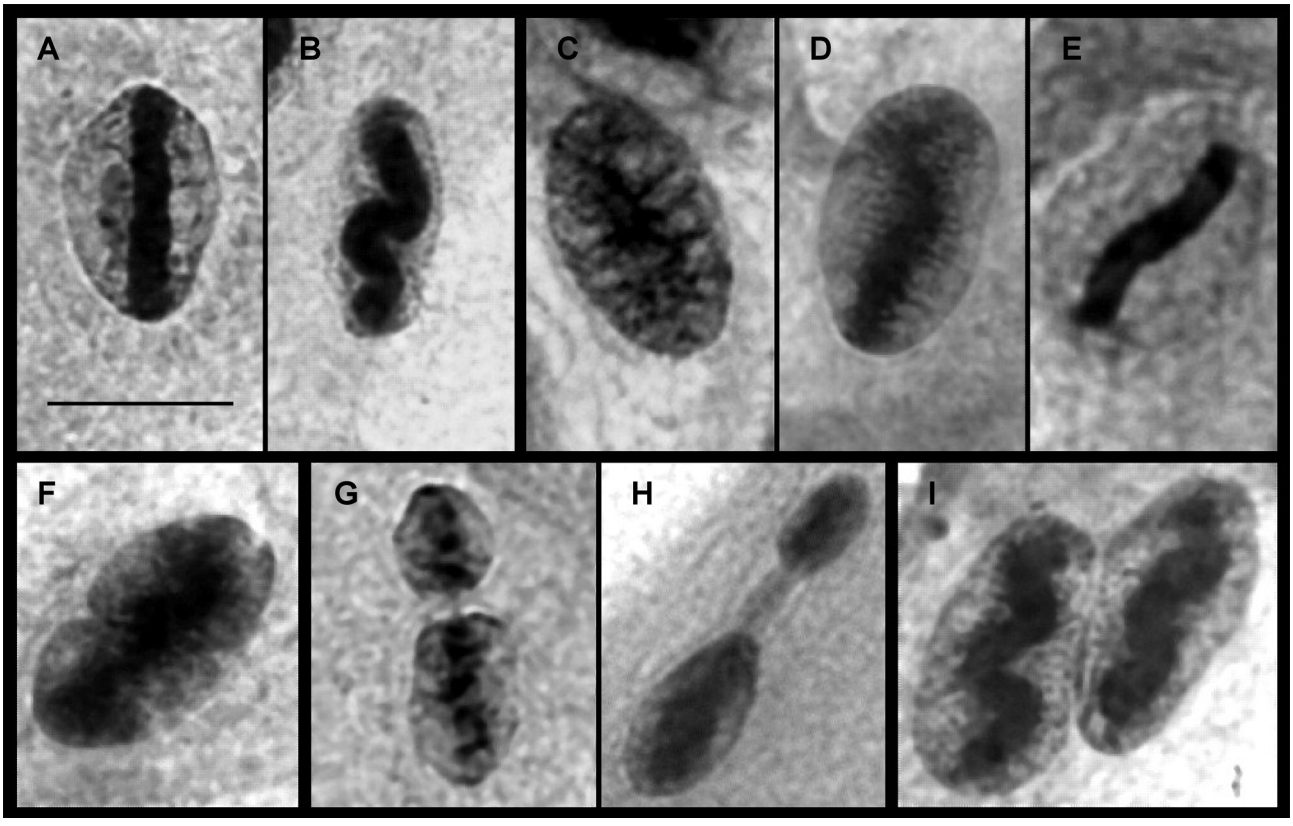

Fig. 2. Light microscopy of several types of SLC structures in epithelial cells from keratoconjunctivitis sicca patients: stick-like **(A)** and snake-like SLC structure **(B)**, early **(C)** and advanced **(D)** stage of chromatin condensation, a fully condensed SLC structure **(E)**, notching of an SLC nucleus **(F)**, binuclear dumb-bell forms with different length chromatin bridges (**G, H**), and a binuclear cell with SLC-structures (**I**). Scale bar: 10 µm.

connected by chromatin bridges. However, it remains to be elucidated whether the binuclear stages of SLC cells connected by chromatin bridges can progress to complete nuclear segregation and subsequent cell division.

The above-mentioned SLC structures, especially the dumb-bell shaped forms, resemble direct division – amitosis. In mammals, amitosis only occurs in highly differentiated tissues (heart, liver, cartilage) as is also the ocular conjunctiva (Yiquan and Binkung, 1986; Tseng, 1989).

We suggest that SLC formation is an attempt at regeneration in pathologically altered tissue, where normal cell division is not allowed to proceed. This

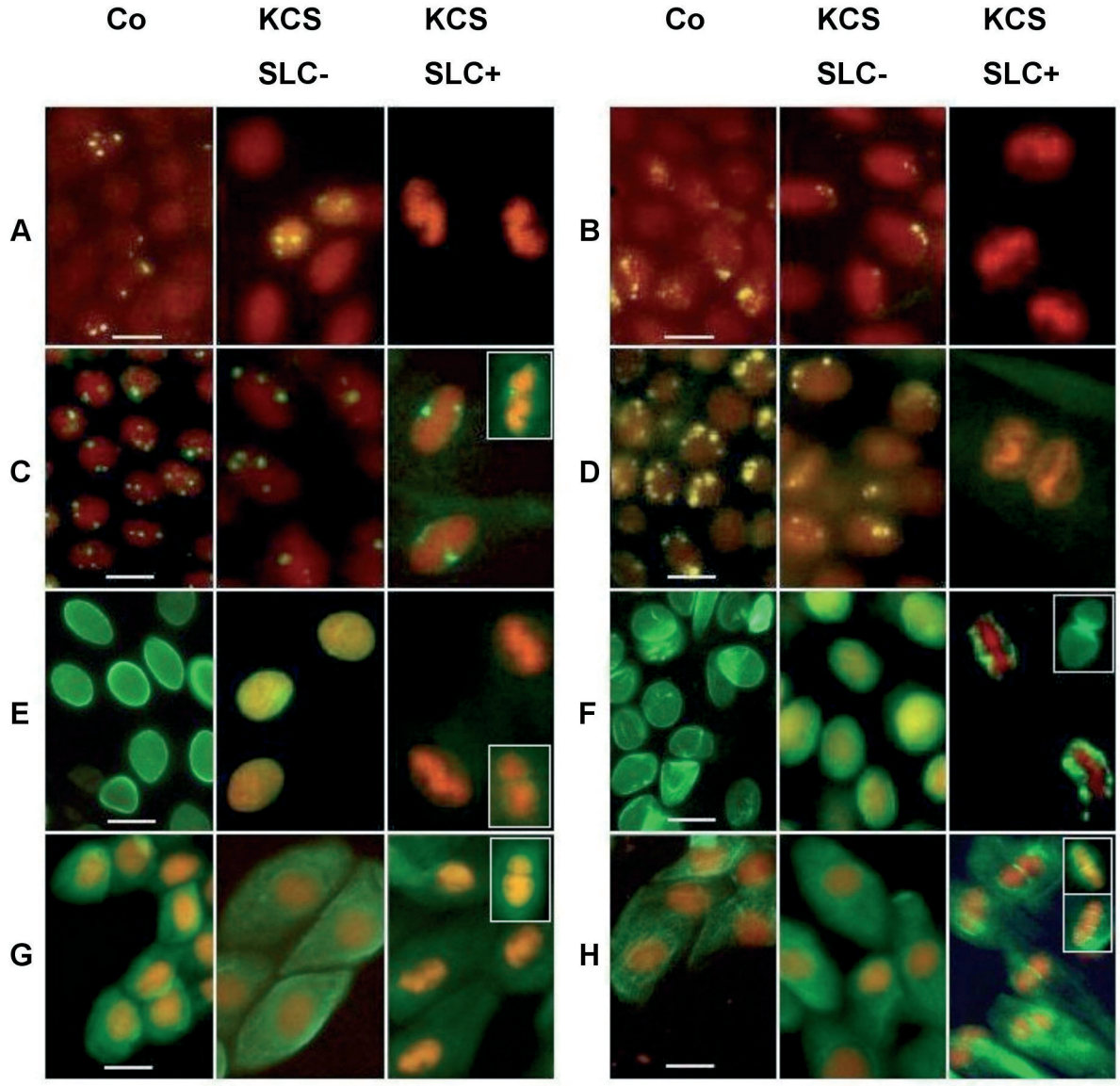

Fig. 3. Immunostaining of human epithelial conjunctival cells of controls (Co) and SLC-negative (SLC-) and SLC-positive (SLC+) cells from KCS patients. Ki-67 (**A**, green) and PCNA (**B**, green) staining were observed in control cells and SLC-negative cells from group 2 but never in SLC-positive cells. Staining for fibrillarin (**C**, green) revealed a characteristic nucleolar pattern in SLC-negative cells. In SLC-positive cells the signal was redistributed in mostly two spots closely adjacent to the central part of the SLC structure, or a weak signal was interspersed along the centre of the SLC structure, (see detail). B-23 (**D**, green) was found in control cells; the number of positive cells and the signal intensity were both decreased in SLC-negative cells of group 2, and the staining disappeared in SLC-positive cells. Lamin A/C immunostaining (**E**, green) revealed strong nuclear membrane positivity in control cells and a weaker signal in SLC-negative cells. The disappearance of any signal was noted in almost all SLC-positive cells, including dumb-bell structures, (see detail). Staining for lamin B (**F**, green) revealed characteristic nuclear membrane positivity in all the examined cells. The signal for pan-cytokeratin (**G**, green) was uniform in the cytoplasm of SLC-negative as well as SLC-positive cells, with no positive pattern in the strangulation belt of dumb-bell structures (see detail). Besides the characteristic cytoplasmic pattern, cytokeratin 14 (**H**, green) was found in the strangulation belt of dumb-bell structures, and the intensity of the signal was more prominent in the locations of nuclear membrane notching, (see detail). Propidium iodide was included in the mounting medium to localize the position of the nuclei (red). Scale bar: 10 µm.

suggestion should be confirmed by direct evidence obtained, for example, in cell culture studies.

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