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Biopsy of the conjunctiva in dry eye patients establishes a correlation between squamous metaplasia and dry eye clinical severity

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PURPOSE. *The aim of this work was to evaluate the different grades of squamous metaplasia of the conjunctiva during the clinical course of dry eye syndrome, detecting the most characteristic morphological and morphometric changes by biopsy in order to provide a diagnostic classification.*
METHODS. *The conjunctiva was studied under light microscopy by conventional histological methods and by morphometric analysis in 165 patients and in 33 controls. Patients were classified according to the Schirmer 1 test, break-up time, rose Bengal staining, osmolarity and impression cytology. The epithelium and connective tissue, with their different cells and other structures, were studied.*
RESULTS. *The conjunctiva in dry eye patients showed progressive stratification, hyperplasia, hypertrophy and cellular flattening, with loss of goblet cell density and mucous layer. We found five pathological grades of squamous metaplasia and one normal grade. Clear nuclear alterations (indentation and binucleation) were found in the early grades of dry eye syndrome, but pyknotic nuclei and anucleated cells were only seen in the most severe grades. The smallest epithelial cells were found in the control group and their size increased with the severity of the dry eye syndrome From the earliest stages to the most severe cases, increases in cellular separation were observed. There was also an increase in the number of inflammatory cells. Blood and lymphatic vessels showed alterations only in the most severe cases.*
CONCLUSIONS. *This is the first grading system proposed for biopsy evaluation of the ocular surface in dry eye patients. These morphological and morphometric studies alone were able, even in the earliest phases of dry eye, to detect the squamous metaplasia that progresses from the surface of the epithelium to the connective tissue. This degenerative or adaptative cellular process was characterized mainly by marked increases in the stratification, epithelial cellular size and a general loss of goblet cells. (Eur J Ophthalmol 2003; 13: 246-56)*

KEY WORDS. *Biopsy, Conjunctiva, Cellular morphology, Dry eye, Ghrading system, Squamous metaplasia*

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INTRODUCTION

The conjunctiva is a part of the tear-producing system of the eye so any alterations may be of diagnostic value in the dry eye syndrome. Patients with dry eye syndromes are usually evaluated in our daily practice on the basis of clinical data, using a clinical grading system (1). However, laboratory and histopathological tests can be helpful in the diagnosis of dry eye syndrome (2). Biopsy is more sensitive than clinical and laboratory tests, and has the advantage of being able to detect squamous metaplasia before keratinisation is clinically detectable (3, 4).

Normal conjunctival epithelium is stratified, secretory, non-keratinised and consists of five cell types (5), and the cells of the superficial layer adhere closely to the preocular tear film. The conjunctival connective tissue is loose with a noticeable fiber component showing glands, lymphatic and blood vessels, and nerve fibers (6). The relationship between the clinical manifestations in dry eye syndrome and histopathology is not clear; but clinically, keratinisation is recognized by slit-lamp biomicroscopic examination, while squamous metaplasia prior to keratinisation can only be demonstrated by morphological techniques (7).

With regard to the diagnostic efficacy of the most frequent clinical tests for dry eye syndrome (8), mainly in the first phases of the disease when symptoms cannot be detected (9), a biopsy permits detailed study of the epithelium and connective tissue, making it possible to compare pathological and non-pathological material, and providing a basis for accurate diagnosis (5). Biopsy can be used to study the whole conjunctival surface and to establish a correlation between the grade of squamous metaplasia and the clinical severity of dry eye (10, 11).

A histopathological classification of the squamous metaplasia on the ocular surface is important because clinicians see patients on the basis of their clinical signs, and classify and treat them according to those signs. A close correlation between the clinical and biopsy stages could increase the value of the clinical grading system. Consequently, we decided to first classify patients according to the clinical stage of the dry eye, and then to look for changes in the ocular surface epithelium. Our histopathological study was based on a clinical grading system (1) closely correlated with an impression cytology grading system (12).

We studied the conjunctival epithelium and connective tissue in dry eye disorder as seen in light microscopy, first, to see how the pathologic changes of the conjunctiva are related to the severity of the clinical symptoms, second, to confirm a differential diagnostic classification; and third, to describe a grading system for the squamous metaplasia during the clinical course of dry eye syndrome, detecting the most characteristic morphological and morphometric changes.

MATERIAL AND METHODS

The patients had al been examined and followed up by the Ophthalmology Service in Ramón y Cajal Hospital and had signed informed consent forms before biopsy specimens were taken. Conjunctival biopsies were performed in 165 patients of both sexes (57.1% females), mean age 53.3 years, and in 33 normal subjects (55% females), mean age 50.2 years. Dry eye syndrome was diagnosed if at least two out of the following three tests were abnormal, according to Pisa's criteria (13): the Schirmer test, tear break-up time (BUT) and rose Bengal vital staining (RB staining) (14). Tear film osmolarity was also evaluated according to Pisa's criteria (13). Impression cytology was rated according to the system for squamous metaplasia given by Murube and Rivas (12). The Schirmer test, BUT and RB staining were all carried out on the same day; osmolarity and impression cytology were done the next day, and biopsy on the third day. Impression cytology and biopsy were taken from nearby sites in the upper-central bulbar area of the conjunctiva under topical anesthesia, 6 mm from the cornea. Biopsy samples of approximately 2 mm^2 were obtained. To study the ocular surface and preserve the tear mucous layer, the material was fixed by immersion in 1.5% glutaraldehyde with tannic acid and HTAB in a buffered solution of cacodylate to pH 7.3-7.4, and post-fixed with 1% osmium tetroxide. The tissue was dehydrated in acetone and embedded in araldite resin (Fluka). Semi-thin sections (1.5 µm) were used. These sections were stained with Richardson methylene blue, and studied in a Nikon 200 light microscope.

Ten fields were photographed from each sample, comprising the outer surface to the connective tis-

sue; less than 1% of cells with nuclear alterations were not included in establishing the percentage of patients with nuclear changes.

The areas of the epithelial cells were measured by image planimetry Pro/Media Cibermetics with a CDA system based on IPP-Plus software. The samples were examined with a Nikon Ophtiphot-2 microscope, attached to a Microflex-DX photograph, and an EVI-1011P color videocamera. We prepared 10 x 15 cm prints at a nominal magnification of 40x. We made a subjective analysis of the general appearance of the cells and estimated the area of the cell nucleus relative to the surrounding cell cytoplasm (N:C ratio). For quantitative studies of conjunctival goblet cells we used a calibrated grid at 400x magnification. The mean total goblet cell density was obtained by counting ten random areas for each specimen. The densities were reported per linear millimeter with standard deviation for each area.

Statistical analysis was done with a statistical package (PRESTA, Clinical Biostatistical Department of Ramón y Cajal Hospital). An unpaired two-tailed Student's test and analysis of variance (ANOVA) were used to compare independent groups; and the non-parametric Spearman's rank correlation coefficient was used to compare two variables. A significant difference was obtained with a probability higher than 95% ($p < 0.05$).

RESULTS

Clinical and laboratory tests are shown in Table I. There were significant differences between each test and the different grades of dry eye syndrome, except in the Schirmer test between grade 1 of dry eye and controls, and in osmolarity, which was similar in grades 4 and 5. The area of non-secretory epithelial cells in the upper bulbar conjunctiva was larger as the severity of the dry eye increased. There were significant differences between all the groups (Tab. II).

The nuclear size of non-epithelial cells in the upper bulbar did not show significant differences between control subjects and grades 1, 2 and 3 of dry eye syndrome. Grades 4 and 5 had a significant decrease of the nuclear size, with very high standard deviations that indicated a high variability of the size (Tab. II).

Nuclear changes were only observed in grades 4 and 5. The cellular area increased with to the severity of the squamous metaplasia. There was a correlation between cellular area and N:C ratio. There were continuous increases from the control subjects to grade 5 of dry eye syndrome, and significant differences between all grades (Tab. II).

The goblet cells became significantly smaller with the severity of the squamous metaplasia. Grade 5 patients had no goblet cells so their size could not be described. The goblet cell density also fell significantly

TABLE I - NUMBER OF PATIENTS WITH ALTERED TEAR TESTS. STATISTICAL DIFFERENCES ARE IN RELATION TO THE PRECEDING GRADE

Test	Control		Dry eye			
	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Schirmer test	1	1	6	10	17	25
	n.s.	*	*	*	*	
Rose Bengal staining	0	2	4	7	26	30
	*	*	*	*	*	
BUT	0	1	5	10	22	28
	*	*	*	*	*	
Osmolarity	0	2	8	12	30	33
	*	*	*	*	n.s.	

n.s. = Not significant; * = $p < 0.05$

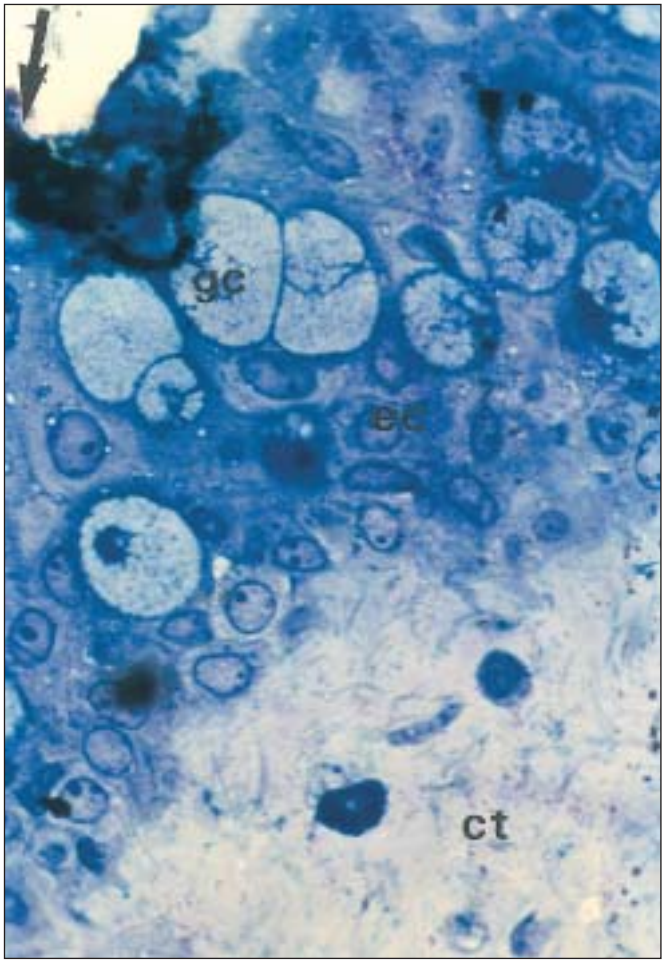

Fig. 1 - *Biopsy from normal subjects (grade 0). Non-secretory epithelial cells (ec) were small. Goblet cells (gc) were numerous, oval and large. Mucin layer was wide (↑). Connective tissue (ct) was loose. Richardson blues staining. Magnification x40.*

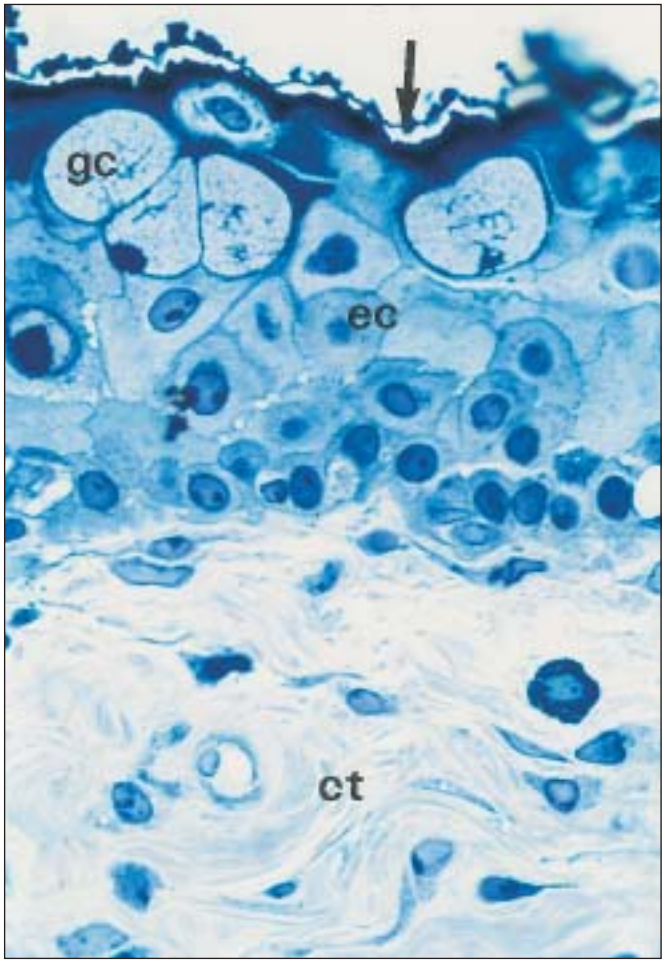

Fig. 2 - *Biopsy from patients with mild dry eye (grade 1). Non-secretory epithelial cells (ec) were slightly separated, and there was a moderate number of goblet cells (gc). Wide mucin layer (↑). Connective tissue (ct) was loose, with few collagen fibers. Richardson blues staining. Magnification x40.*

with the increase in the severity of the squamous metaplasia. Goblet cells in normal eyes and grades 1 and 2 of dry eye were arrayed over the whole epithelium but in grade 3 and in patients with primary Sjögren´s syndrome they had disappeared in the deepest layers. The scarce goblet cells in grade 4 were only in the central layers. Patients with grade 5 had no goblet cells (Tab. III).

The connective tissue showed a significant, progressive increase of collagen fibers from normal subjects to grade 5 dry eye syndrome. These fibers were loose in normal subjects and in grade 1 patients. The connective tissue was moderately dense in grade 2 patients. There was an abundance of compactly arranged fibers in the conjunctivae of grade 3 patientsand in grades 4 and 5 the connective tissue was extremely dense.

The number of inflammatory cells in the connective tissue increased with the severity of the disease. A few fibroblasts and macrophages were seen in the controls. Conjunctivae with grades 1 and 2 of dry eye had a slight increase in these cells. The most common cell types in grade 3 conjunctivae were abundant fibroblasts, macrophages, with a few mast cells, wandering lymphocytes and plasma cells. The numbers of these cell types increased with the clinical severity.

The mucous layer from tear film was fixed to the superficial cells for morphological study. The width and distribution continuously decreased from grade 0 to grade 5. Grade 3 showed a very thin, discontinuous mucous layer and grades 4 and 5 usually had no mucous layer. These findings are summarized in Table IV.

DISCUSSION

The six clinical severity groups (1) and six grades of squamous metaplasia by impression cytology used in this study (12) are summarized in Table V. Each grade had a complete relation with our histoplathological results. Thus, patients with clinical grade 0 had the same grade of squamous metaplasia by impression cytology and biopsy. Likewise, clinical grade 1 was correlated with morphological grade 1; patients with clinical grade 2 had grade 2 squamous metaplasia, and so on. Conjunctival biopsy is therefore usually useful for an accurate diagnosis of certain tear deficiency states. It may also be helpful in patients with clinical evidence of systemic disease. In ocular pemphigoid, Stevens-Johnson syndrome or primary Sjögren´s syndrome, biopsy specimens can show morphological changes, although repeated biopsies may be necessary to firmly establish the diagnosis. In general, the morphological changes in the conjunctival

TABLE II - NON-SECRETORY EPITHELIAL CELLS IN THE UPPER-CENTRAL BULBAR CONJUNCTIVA. CELLULAR AND NUCLEUS AREAS IN μm^2. Statistical differences are in relation to the preceding grade

	Control Grade 0		Grade 1		Grade 2		Dry eye Grade 3		Grade 4		Grade 5
Cellular area	110.1±30.7	*	180.3±26.2	*	269.4±34.4	*	383.1±44.9	*	502.1±42.6	*	620.7±51.1
Nuclear area	37.7±10.1	n.s.	38.1±9.0	n.s	39.0±11.8	n.s	38.2±9.9	n.s	30.1±12.6	n.s	23.9±21.3
N:C ratio	1:3	*	1:5	*	1:7	*	1:10	*	1:18	*	1:30

n.s. = Not significant; * = p< 0.05

TABLE III - GOBLET CELLS IN THE UPPER-CENTRAL BULBAR CONJUNCTIVA. DENSITY DESCRIBED IN CELLS/mm^2±SD AND CELL AREA IN μm^2. Statistical differences are in relation to the preceding grade

	Control Grade 0		Grade 1		Grade 2		Dry eye Grade 3		Grade 4		Grade 5
Cellular area	267.2±25.6	*	221.1±22.0	*	170.5±20.4	*	124.6±15.0	*	16.3±12.5	*	0
Density	>100		100-50		50-25		25-5		<5		0
Disposition	Along the whole epithelium		Along the whole epithelium		Along the whole epithelium		Central and superficial layers		Central layers		-

n.s. = Not significant; * = p< 0.05

epithelium in dry eye syndrome are well known, but the relationship between the patient's clinical condition and conjunctival morphology is obscure (15, 16).

There is a correlation between pathological findings in the conjunctiva and the severity of the clinical symptoms in dry eye syndrome. As regards the diagnostic efficacy of the most frequent ocular tests for dry eye syndrome, changes may appear before the clinical signs

TABLE IV - SUMMARY OF THE MORPHOLOGICAL RESULTS

	Control			**Dry eye**		
	Grade 0	**Grade 1**	**Grade 2**	**Grade 3**	**Grade 4**	**Grade 5**
			Goblet cells			
Density (number/mm^2)	> 100	100 to 50	50 to 25	25 to 5	< 5	None
Cellular area	270 µm^2	280 µm^2	220 µm^2	160 µm^2	16 µm^2	-
Mucin content	Plenty	Plenty	Less plentiful	Very few	Empty	-
			Mucous layer			
Size	Very high	Less high	Narrow	Discontinuous	None	None
Arrangement	Continuous	Continuous	Continuous	In clots	Small clots	-
			Non-secretory cells			
Cell size	110 µm^2	180 µm^2	270 µm^2	380 µm^2	500 µm^2	620 µm^2
Cytoplasm	Normal	Normal	Normal	Less stained	Mild keratinisation	Usually keratinised
Nuclear size	38 µm^2	38 µm^2	39 µm^2	38 µm^2	30 µm^2	24 µm^2
Nuclear changes	None	None	Indentations binucleation	Indentations binucleation pyknosis	Pyknosis anucleated	Frequently absent
N:C ratio	1:3	1:5	1:7	1:10	1:18	1:30
Cellular arrangement	In sheets	In sheets	Slightly separated	Very separated	More separated	Completely separated
			Connective tissue			
Collagen fibers	Loose	Loose	Denser	Very dense	Markedly dense	Completely dense
Cell density	Normal	Normal	More fibroblasts	Many fibroblasts and inflammatory cells	More fibroblasts and inflammatory cells	Even more fibroblasts and inflammatory cells
Lymphatic and blood vessels	Normal	Normal	Slightly altered	Moderately altered	Markedly altered	Very altered, often occluded

(8). When dry eye syndrome is suspected, manly in the first phases of the disease, biopsy allows a precise study of the ocular surface and tear film mucous, making it possible to compare pathological and non-pathological material (9, 15) and permitting an accurate diagnosis (16). A biopsy can be used to study the whole conjunctival surface from the epithelium to the connective tissue, and thus establish the severity of dry eye. A conjunctival biopsy also distinguishes between an extrinsic and intrinsic syndrome (17).

We have found a continuous process of squamous metaplasia in non-systemic disorders. Grade 0 corresponds to a normal tear film and ocular surface, grade 1 to a slightly altered tear film and normal ocular surface. Grade 2 has a moderately altered tear film, slightly altered epithelium, and completely normal connective tissue, grade 3 an altered tear film, moderately modified epithelium and slightly altered connective tissue. Grade 4 had a completely altered tear film and epithelium, and a moderately altered connective tissue, grade 5 a very markedly modified tear film and ocular surface.

Non-systemic disorders are frequently mild to start with (grade 1) and may gradually progress. Grade 5 is rare as the syndrome must be very severe or the alteration chronic, so grades 4 and 5 usually appear in intrinsic diseases (15, 16). F rom this information, we propose the following grading system to diagnose a dry eye syndrome by conjunctival biopsy:

Grade 0

Non-secretory cells are small (around 110 μm^2) with large, round nuclei (around 38 μm^2). The N:C ratio is around 1:3. The tear mucous layer is very high. Epithelial cells are joined, forming a sheet, with very small, tightly closed spaces between them. The goblet cells are plentiful (more than 100 cells/linear µm), large (around 270 μm^2), oval, and their cytoplasm contains plenty of mucin. Connective tissue is loose (Fig. 1).

TABLE V - CHARACTERISTICS OF THE CLINICAL GRADING SYSTEM AND THE GRADES OF IMPRESSION CYTOLOGY USED IN THIS STUDY

	Grade 0	**Grade 1**	**Grade 2**	**Grade 3**	**Grade 4**	**Grade 5**
			Clinical grading system			
Grade	Normal	Normal	Mild dryness	Moderate dryness	Severe dryness	Visual deficiency
Symptoms	No	Only under overexposure	In daily life	In daily life	In daily life	In daily life
Clinical signs	No	No	No	Reversible	Irreversible	Irreversible
			Impression cytology grading system			
Goblet cell density	>400 cells/mm^2	400-300 cells/mm^2	300-200 cells/mm^2	200-50 cells/mm^2	50-10 cells/mm^2	<5 cells/mm
Goblet cell staining	PAS-positive	PAS-positive	PAS-positive	Slight PAS-positive	PAS-negative	PAS-negative
Non-secretory cell size	200-300 μm^2	200-350 μm^2	350-500 µm	500-900 μm^2	900-1600 μm^2	> 1600 μm^2
N:C ratio	1:1-1:2	1:3-1:4	1:5-1:6	1:10	1:20	1:30
Cellular arrangement	Perfectly joined	Usually joined	Moderately separated	Frequently separated	Frequently isolated	Isolated
Main nuclear type	Normal	Normal	Binucleated	Binucleated or pyknotic	Pyknotic or anucleated	Anucleated

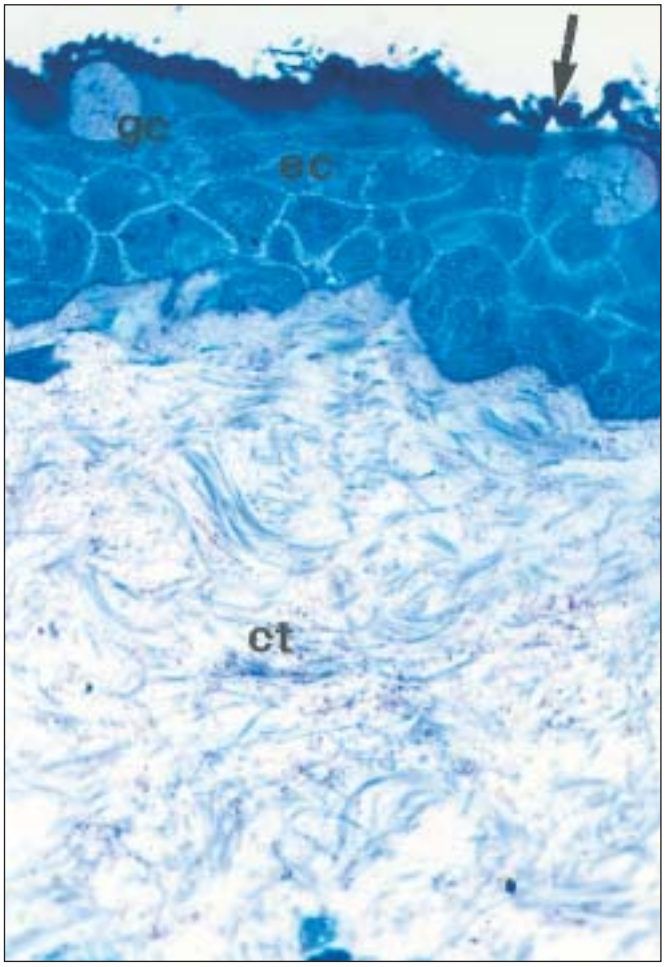

Fig. 3 - *Biopsy from patients with moderate dry eye (grade 2). Non-secretory epithelial cells (ec) were moderately separated, and there was a smaller number of goblet cells (gc), with PAS-positive cytoplasm. Connective tissue (ct) was denser and the mucin layer less wide (↑). Richardson blues staining. Magnification x40.*

Grade 1

The epithelial cells are larger (around 180 μm^2). There is slight reduction of the height of the mucous layer. The nuclei have a similar size (around 38 μm^2) with slight invaginations of the membrane and bigger spaces between them. The N:C ratio is around 1:5. The goblet cells are moderate in number (between 100 and 50 cells/linear mm), maintaining their size (around 280 μm^2) and their oval shape, and give an intense stain. Connective tissue is slightly denser, with a higher concentration of collagen fibers and cells (Fig. 2).

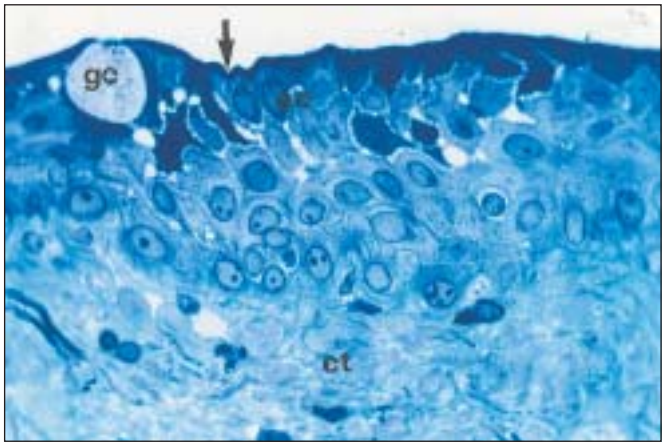

Fig. 4 - *Biopsy from patients with moderate-severe dry eye (grade 3). Non-secretory epithelial cells (ec) were more separated, and there were very few goblet cells (gc). The mucin layer (↑) was very thin and discontinuous. Connective tissue (ct) was moderately dense. Richardson blues staining. Magnification x40.*

Grade 2

Epithelial cells are larger (around 270 μm^2), elongated and flattened. The tear mucous layer is narrow but complete. The nuclei are of a similar size (around 39 μm^2) with deep indentations of the membrane, and may be binucleated. The N:C ratio is around 1:7. The intercellular spaces are often expanded. The number of goblet cells is markedly lower (between 50 and 25 cells/linear mm), with less mucin, and are usually smaller (around 220 μm^2). The connective tissue is slightly denser. Lymphatic and blood vessels are usually slightly altered. In general, there is a slight increase of inflammatory cell density (Fig. 3).

Grade 3

The epithelial cells are larger (around 380 μm^2), elongated and flattened. Nuclei are of similar size (around 38 μm^2), with deeper indentations of the membrane, and usually present binucleation, pyknosis and anucleation. The N:C ratio is around 1:10. There is only a small number of goblet cells (between 25 and 5 cells/linear mm) and their size is quite different (around 160 μm^2), as is the shape, with the cytoplasm almost devoid of mucin. The connective tissue is denser, with abundant fibroblasts, lymphocytes, macrophages, mast and plasma cells. Lymphatic and blood vessels are moderately altered (Fig. 4).

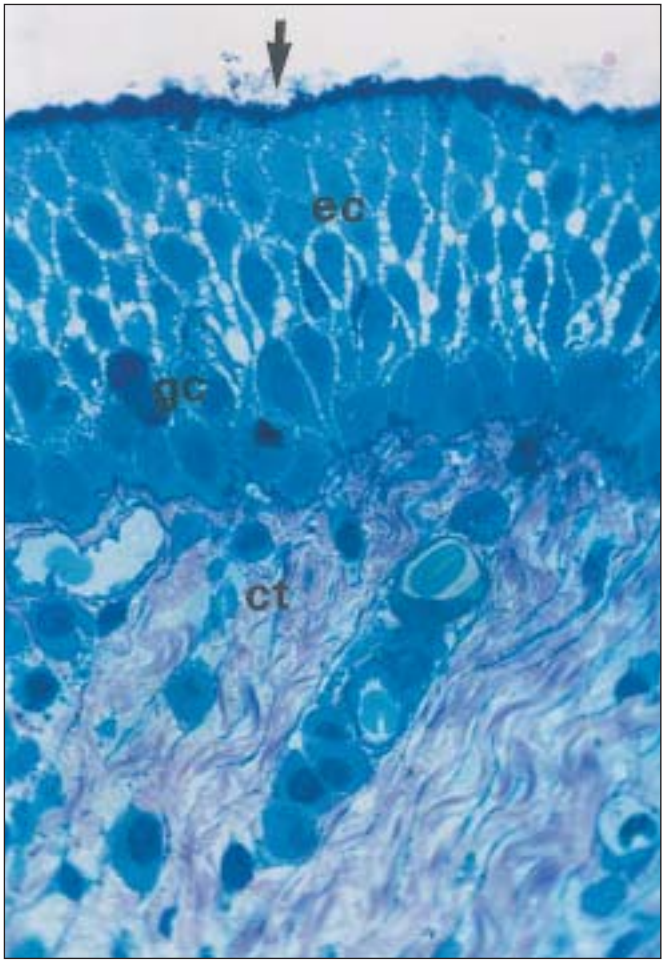

Fig. 5 - *Biopsy from patients with severe dry eye (grade 4). Non-secretory epithelial cells (ep) were flattened, keratinized and very separate, with nuclear alterations, or frequently absent altogether. Goblet cells had usually disappeared. The mucin layer (↑) had disappeared completely. Connective tissue (ct) was very dense. Richardson blues staining. Magnification x40.*

Fig. 6 - *Biopsy from patients with severe-plus dry eye (grade 5). Non-secretory epithelial cells were keratinized, at least the most superficial layers. There were no goblet cells. The mucin layer had disappeared completely. Connective tissue was very dense. Richardson blues staining. Magnification x40.*

Grade 4

The epithelial cells are very large (around 500 μm^2), elongated and flattened, with a mild degree of keratinisation. Nuclei are very small (around 30 μm^2), frequently pyknotic and sometimes absent. The N:C ratio is about 1:18. The intercellular spaces are more expanded. Usually, there are very few goblet cells (fewer than 5 cells/linear mm) and they are completely altered in size (around 16 μm^2) and shape, without any mucin. The connective tissue is markedly dense, with large numbers of fibroblasts, lymphocytes, macrophages, mast cells and plasma cells. Lymphatic and blood vessels are markedly altered (Fig. 5).

Grade 5

The epithelial cells are very large (around 620 μm^2) and usually keratinized. Nuclei are frequently absent, and are sometimes pyknotic (around 24 μm^2) with an N:C ratio of 1:30. The intercellular spaces are more expanded. There are no goblet cells. The connective tissue is completely dense, with a larger number of inflammatory cells. Lymphatic and blood vessels are very altered, often occluded (Fig. 6).

All tissue specimens can be studied by conjunctival biopsy and impression cytology, and these methods are helpful in the diagnosis of dry eye syndrome,

as both are extremely sensitive (17, 18). Although these techniques are very different they provide important, complementary results.

Impression cytology is a non-invasive method, performed under local anesthesia. It permits the diagnosis of a dry eye disorder by only examining the most superficial layers of the conjunctival epithelium, and studying the general characteristics of the cell. Impression cytology has been adapted for use in everyday practice. The results are immediate. Conjunctival biopsy also only needs topical anesthesia. Only pinhead-sized tissue specimens are required, with minimal discomfort for patients. This method permits an exact three-dimensional structural diagnosis because the conjunctival sample is preserved; so both epithelium and connective tissues can be observed.

Biopsy is not routinely done for the diagnosis of dry eyes, because it is time-consuming (18). It gives more information than impression cytology, but impression cytology takes less time. Depending on the needs, one or the other has to be performed. As these techniques are complementary, we think they should both be done in cases of systemic disorders.

In summary, the diagnosis of squamous metaplasia can be confirmed by biopsy, mainly in doubtful clinical diagnosis. We feel it should be used as a prognostic tool for assessing the ocular surface in patients with dry eye syndrome associated with a systemic disorder, as it is the most important ocular test for a differential diagnosis. Our results indicate that the process of squamous metaplasia can serve as a basis for a grading system of squamous metaplasia using morphological and morphometric parameters to describe six statistically different grades, corresponding to the clinical grading system.

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REFERENCES

1. Murube J. Clasificación clínica del ojo seco. In: Murube J, ed. Ojo Seco-Dry Eye. Tecnimedia Editorial. Madrid, 1997; 39-44.
2. Oroza MA. Estudio inmunohistoquímico y morfológico en ojos de pacientes con síndrome de Sjögren. Doctoral Thesis. University Complutense of Madrid. June 2000. (Quoted in: Rivas L, Murube J. Utilización de la citología de impresión en el diagnóstico de las enfermedades de la superficie ocular. Novartis Ophthalmics. Barcelona. Spain, 2001: 1-33.
3. Nelson JD, Wright JC. Conjunctival goblet cell densities in ocular surface disease. Arch Ophthalmol 1984; 102: 1049-51.
4. Rivas L, Oroza MA, Pérez-Esteban A, Murube J. Topographical distribution of ocular surface cells by the use of impression cytology. Acta Ophthalmol 1991; 69: 371-6.
5. Steulh KP. Ultrastructure of the conjunctival epithelium. In: Straub W, ed. Developments in Ophthalmology. Basel: Karger 1989; 19: 1-104.
6. Lemp MA, Marquardt R. Morfología funcional de la conjuntiva. El Ojo seco. Barcelona: Springer-Verlag Ibérica 1994; 2: 35-61.
7. Tseng SCG. Topical tretinoin treatment for dry-eye disorders. Int Ophthalmol Clin 1987; 27: 47-53.
8. Roberts DK. Keratoconjunctivitis sicca. J Am Optom Assoc 1991; 62: 187-99.
9. Rivas L, Álvarez MI, Rodríguez JJ, Murube J. Ophthalmological tests in patients with and without association of primary Sjögren's syndrome. Ger J Ophthalmol 1995; 4: 306-10.
10. Rivas L, Toledano A, Álvarez MI, Sanz AI, Oroza MI, Murube J. Ultrastructural study of the conjunctiva in patients with keratoconjunctivitis sicca not associated with systemic disorders. Eur J Ophthalmol 1998; 8: 131-6.

11. Rivas L. Pruebas histológicas en el ojo seco. In: Murube J, ed. Ojo seco-Dry eye. Madrid: Tecnimedia Editorial SL, 1997: 159-67.
12. Murube J, Rivas L. Impression cytology on conjunctiva and cornea in dry eye patients establishes a correlation between squamous metaplasia and dry eye clinical severity. Eur J Ophthalmol 2003; 13: 115-27.
13. Murube del Castillo J, Cortés Rodrigo MD. Eye parameters for the diagnosis of xerophthalmos. Clin Exp Rheumatol 1989; 7: 145-50.
14. Van Bijsterveld OP. Diagnostic tests in the sicca syndrome. Arch Ophthalmol 1969; 82: 10-4.
15. Seamone CD, Jakson WB. Sjögren's syndrome. In: Tasman W, ed. Immunology of the external eye. Duane's Clinical Ophthalmology. Philadelphia: Lippincott-Raven Publishers; 1997. Vol. IV, chapter 2.
16. Wilson RW, Sexton RR. Laboratory aids in diagnosis. In: Tasman W, ed. Duane's Clinical Ophthalmology. Philadelphia: Lippincott Raven Publishers;1996. Vol IV, chapter 1.
17. Rivas L, Oroza, Pérez-Esteban A, Murube J. Topographical distribution of ocular surface cells by the use of impression cytology. Acta Ophthalmol 1991; 69: 371-6.
18. Nelson JD. Diagnostico de la keratoconjuntivitis sicca. In: Friedlaender M, ed. International Ophthalmology Clinics. Ojo seco. Buelach: Little, Brown and Company Inc. CibaVision SA, 1995: 37-56.