International Scholarly Research Network
ISRN Pathology
Volume 2012, Article ID 359421, 7 pages
doi:10.5402/2012/359421

Research Article

Progressive Extracellular Matrix Disorganization in Chemically Induced Murine Oral Squamous Cell Carcinoma

B. Fuentes,[1] J. Duaso,[2] D. Droguett,[1,2] C. Castillo,[2] W. Donoso,[1] C. Rivera,[3] B. Venegas,[1] and U. Kemmerling[2]

[1] *Departamento de Estomatología, Facultad de Ciencias de la Salud, Universidad de Talca, VII Region, Talca, Chile*
[2] *Programa de Anatomía y Biología del Desarrollo, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Región Metropolitana, Santiago de Chile 8380453, Chile*
[3] *Departamento de Ciencias Básicas Biomédicas, Facultad de Ciencias de la Salud, Universidad de Talca, VII Region, Talca, Chile*

Correspondence should be addressed to U. Kemmerling, ukemmerling@med.uchile.cl

Received 4 July 2012; Accepted 29 July 2012

Academic Editors: A. B. Galosi and P. Paglini-Oliva

Introduction. Oral squamous cell carcinoma (OSCC) is one of the ten most common cancers affecting the human population. Tumor pathogenesis implies a multistep process in which cells acquire features that enable them to become tumorigenic and ultimately malignant. The process of OSCC carcinogenesis can be reproduced in animal models, the OSCC induction with 4-nitroquinoline-1-oxide (4NQO) in mice is a widely used tool for studying tumor pathogenesis. *Objective*. The aim of the present study was to determine the progressive changes in basal lamina and connective tissue remodeling during 4NQO-induced OSCC carcinogenesis. *Material and Methods*. Samples were classified according to "International Histological Classification of tumors" in mild, moderate, and severe dysplasia and invasive carcinoma. Five samples of each pathologic entity and control healthy tongues were used. Immunohistochemical analysis of collagen IV as well as histochemical analysis of glycosylated molecules (PAS) and collagen I (Picro Sirius red) were performed. *Results*. During experimental-induced carcinogenesis by 4NQO a progressive basal lamina destruction and collagen I disorganization in adjacent connective tissue can be observed. *Conclusion*. Our results confirm previous studies that show alterations in extracellular matrix (ECM) in malignant lesions, validating the experimental carcinogenesis induced by 4NQO.

1. Introduction

Squamous cell carcinoma of the oral cavity (OSCC) is one of the ten most common tumors affecting the human population [1]. Continued population growth and aging have contributed to the increase in this type of pathology, making it an important public health problem. It starts as an epithelial dysplasia and is characterized by an altered proliferation of squamous dysplastic cells of the epithelial surface stratum [2]. It progresses to degrade the subepithelial basement membrane [2, 3], generating a local destruction and distant invasion through the process of metastasis [3–5]. Local invasion of the underlying connective tissue occurs through islets and cords of epithelial cells [3, 6].

Interaction between tumor cells and extracellular matrix (ECM) components is essential for tumor growth [7, 8] and for the onset of cell spreading and subsequent metastatic activity [9].

The basal lamina, a physicochemical barrier to tumor invasion [10], plays a fundamental role in the processes described above. It is a highly specialized structure, consisting of a set of molecules with different sensitivity to proteolytic degradation. Its components are synthesized and secreted by epithelial cells and connective tissue [11]. They form a dense network of collagen IV [9] and other macromolecular components such as proteoglycans, glycoproteins, and glycosaminoglycans as well as laminin, fibronectin, and tenascins.

Collagen IV is one of the most affected molecule, its degradation during tumor invasion is mediated by metalloproteinases secreted by neoplastic cells. In advanced stages of invasion or in lesions with a greater degree of malignancy, the

capacity of degradation is further increased [9], associating the discontinuity of the basal lamina with an increased probability of metastasis and a poor prognosis [12].

The interactions between the neoplastic cells and components of the ECM in the surrounding connective tissue [13] as well as changes the basement membrane influences tumor behavior during the invasion process [14].

Once fully or partially degraded the basal lamina, neoplastic cells interacts with the ECM of connective tissue, in which collagen I is disorganized, facilitating in that way the tumor invasion [5].

Carcinogenesis and its different stages can be reproduced in animal models by chemical induction. The different experimental models of carcinogenesis are fundamental tools for studying the pathogenesis of cancer. The induction of OSCC with 4-nitroquinoline-1-oxide (4NQO) in mice is one of the most widely used animal models [15]. 4NQO generate reactive oxygen (ROS) and nitrogen (RNS) species [16], which directly and indirectly induce DNA adducts and oxidize other macromolecules like proteins and lipids [16]. In this model, the murine oral epithelium undergoes pathologic changes from mild dysplasia to invasive carcinoma.

The aim of the present study was to determine ECM changes in basal lamina and connective tissue during chemically induced carcinogenesis in mice.

2. Materials and Methods

2.1. Animals and Carcinogen Treatment. Six-week-old female CF-1 mice, purchased from the Public Health Institute of Chile, were used. Animals were fed on certified Champion diet with free access to water under standard conditions of 12 h dark-light period. The treatments were carried out as described previously [15]. The carcinogen 4-NQO (Sigma, St. Louis, MO, USA) stock solution was prepared weekly in propylene glycol at 5 mg/mL and stored at 4°C. The 4-NQO stock solution was diluted in the drinking water (26 mM) receiving each mouse a dose of 12 mg/kg/day. The water was changed weekly. The mice were randomly divided into an experimental group in which the drinking water contained 4-NQO (20 animals) and a control group (10 animals) in which the drinking water contained no 4-NQO, only the same volume of propylene glycol. After a 16-week carcinogen treatment, mice were analyzed clinically for precancerous and cancerous lesions in the oral cavities at different times for up to 12 weeks or until signs of sickness or weight loss. The experimental protocol was approved by the Universidad de Talca Institutional Animal Care and Use Committee, which follows the recommendations of the Canadian Council on Animal Care [17].

2.2. Histological and Histochemical Techniques. The tongues of mice were dissected immediately after cervical dislocation. Cross-sections of the tongues were cut and fixed in 10% formaldehyde in 0.1 M phosphate buffer (pH 7.3) for 24 h, then dehydrated in alcohol, clarified in xylene, embedded in paraffin, and sectioned at 5 μm. Paraffin histological sections were stained with hematoxylin-eosin for routine histological analysis, Picrosirius red-hematoxylin for collagen histochemistry [18], and periodic acid-Schiff (PAS) for carbohydrate containing tissue elements (Sigma-Aldrich kit 395B). As a control of PAS method, 5 μm-thick sections were incubated with a 4 μg/mL solution of α-amylase (Nutritional Biochemical Corporation) in PBS pH 6.0, for 30 minutes at 37°C prior to the PAS-hematoxylin reaction. A decrease in the intensity of the PAS stain reaction after the enzyme treatment was considered as evidence of the presence of carbohydrates.

2.3. Immunohistochemistry. The tongues were processed as described above and standard immunoperoxidase techniques were used to show collagen IV (Novocastra NCL-COLL - IV dilution 1 : 500 v/v). The primary antibody was applied individually to each section overnight at 4°C. Immunostaining was performed using a horseradish peroxidase-labelled streptavidin biotin kit (RTU-Vectastain kit) following the manufacturer's directions using diaminobenzidine as the chromogen. Sections were counterstained with Mayer's haematoxylin (DAKO) and mounted with Entellan (Merck). Immunohistochemical controls were done by replacing the primary antibodies with phosphate buffered saline. All controls were negative. All sections were examined by light microscopy (Leitz Orthoplan), ten fields were selected randomly and the signal intensity was scored as follows: −, absent; +/−, patchy; +, weak; ++, moderate; +++, high [19]. Images were captured with a Canon 1256 camera.

2.4. Histological Classification of Lesions. Tongue lesions were classified according to the "WHO International Histological Classification of Tumors" in mild, moderate, and severe dysplasia/carcinoma in situ or invasive OSCC [20].

3. Results

3.1. During 4NQO Carcinogenesis Basal Lamina between Epithelia and Connective Tissue Is Progressively Destroyed. Histological sections of the different tongue lesions (mild dysplasia to invasive carcinoma) stained with the histochemical PAS method (Figure 1). PAS reagent is mainly used for staining structures containing a high proportion of carbohydrate macromolecules (glycogen, glycoprotein, and proteoglycans), typically found in connective tissues and basal lamina [21]. Basal lamina forms a barrier that the malignant lesion must destroy to invade the underlying connective tissue. Control lingual mucosa samples, from not 4NQO treated mice, shows a thick, continuous, and homogeneous basal lamina (Figures 1(a)-1(b), arrow). Mild (Figures 1(c)-1(d)) and moderate (Figures 1(e)-1(f)) dysplasia shows also a continuous and homogenous basal lamina similar to that of the normal lingual mucosa. In severe dysplasia (Figures 1(g)-1(h)), basal lamina continuity is maintained, but is thinner than in control mucosa or with lower grade dysplasia. In OSCC (Figures 1(i)-1(j)) the basal lamina is thinner and irregular, showing loss of continuity in several points through its length (Figure 1(j) arrow). The continuity

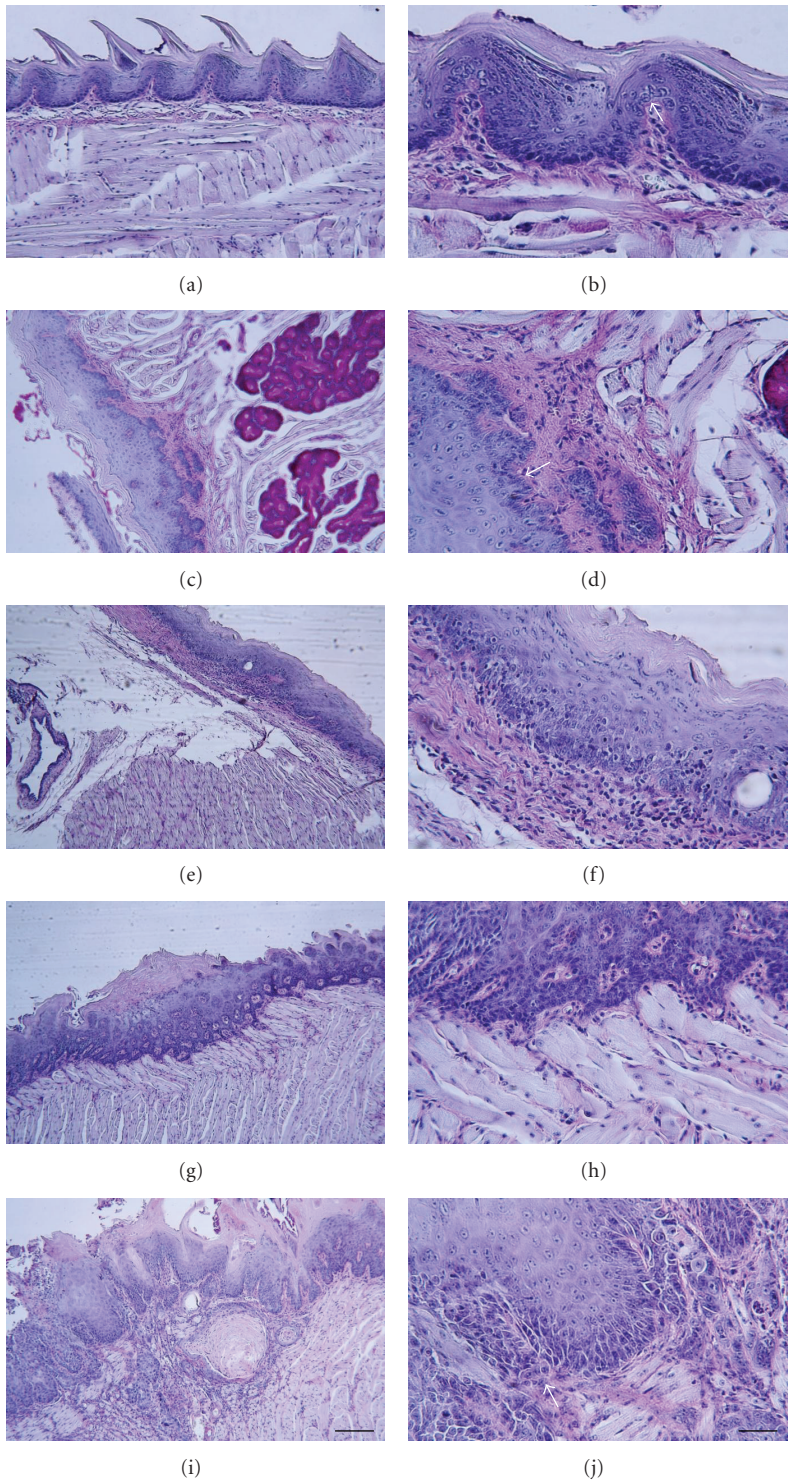

Figure 1: During 4NQO carcinogenesis basal lamina between epithelia and connective tissue is progressively destroyed (PAS histochemistry): Histological sections of control, healthy tongue ((a)-(b)), mild ((c)-(d)), moderate ((e)-(f)), and severe ((g)-(h)) dysplasia as well as OSCC ((i)-(j)) were stained with the PAS method for the detection of glycosylated components. PAS reactivity is progressively loss and completely absent in areas of OSCC, particularly in areas of connective tissue invasion. Bar scales: 50 μm ((a), (c), (e), (g), and (i)), 25 μm ((b), (d), (f), (h), and (j)).

of the basal lamina is completely lost in those areas where the OSCC cells invade the connective tissue.

Cells from squamous cell carcinomas secret collagenases that are able to degrade the collagen IV present in the basal lamina. The loose of Collagen IV immunoreactivity is used to study the tumor biological behavior [22]. The progressive destruction of the basal lamina during 4NQO-induced carcinogenesis evidenced by the PAS staining is also observed by collagen IV immunoreactivity. Tongues from control lingual samples and with mild dysplasia show strong collagen IV immunoreactivity (+++) (Figure 2(a)), which became progressively loose in the moderate dysplasia (Figure 2(c)) and is patchy (−/+) or completely absent (−) in severe dysplasia (Figure 2(d)), and areas where the OSCC invades the connective tissue (Figure 2(e)). Cells from the basal layer in normal mucosa and mild dysplasia are surrounded by a continuous line which is positive for collagen IV (Figures 2(a)-2(b)), which disappear in the more severe lesions (Figures 2(c)-2(e)).

3.2. During 4NQO Carcinogenesis the ECM Connective Tissue is Progressively Remodeled. One of the principal fibrous components of the ECM of connective tissue is collagen I [23]. The histochemical picrosirius red method stains the collagen I fibers in red and the collagen III fibers in green [18]. Control lingual mucosa samples, from not 4NQO treated mice, shows a subepithelial connective tissue in which collagen I fiber predominates (Figure 3(a)). In mild (Figure 3(b)) and moderate (Figure 3(c)) dysplasia collagen I fibers also predominates. However, in severe dysplasia (Figure 3(d)) and OSCC (Figure 3(e)) an increasing staining for collagen III fibers can be observed.

4. Discussion

The ECM is a key regulator of cell and tissue function. Traditionally, the ECM has been thought of primarily as a physical scaffold that binds cells and tissues together [23]. However, the ECM is a dynamic structure that interacts with cells and generates signals through feedback loops to control the behavior of cells. Thus, ECM macromolecules are bioactive and modulate cellular events such as adhesion, migration, proliferation, differentiation, and survival [24]. Additionally, ECM molecules are strictly organized and this organization determines the bioactivity of the ECM. Even minor alterations in a single ECM component can lead not only to altered physicochemical properties of tissues but also to changes in cellular phenotype and cell-matrix interactions. It has been proposed that these changes in ECM structure and bioactivity in tissue function ultimately lead to development of disease [25].

Neoplastic cells change host microenvironment that undergoes extensive change during the evolution and progression of cancer. This involves the generation of cancer-associated fibroblasts, which, through release of growth factors and cytokines, lead to enhanced angiogenesis, increased tumor growth and invasion. The altered fibroblast phenotype also contributes to the development of an altered ECM. The inflammatory infiltrate associated with many solid tumors also modulates tumor function, having both anti- and protumor effects [26]. Cancer cell migration and invasion into adjacent connective tissue depends on ECM. Proteolysis of the ECM regulates cellular migration by modifying the structure of the ECM scaffold and by releasing ECM fragments with biological functions. ECM proteolysis is therefore tightly controlled in normal tissues but typically deregulated in tumors [23]. Changes in ECM in areas of invasive tumor front (ITF) are used in some systems to classify neoplastic lesion of epithelia origin, like the Bryne's multifactorial grading system for the ITF [27]. The basal lamina, a highly specialized structure of ECM, has traditionally been described as the first barrier to be crossed by malignant epithelial neoplastic cells to invade the underlying connective tissue [10]. Basal lamina components are degraded by proteases secreted by action of tumor and host cells. Between the most studied proteases involved in ECM destruction are the matrix metalloproteinases (MMPs), specifically MMP-2 and MMP-9 [14].

The OSCC induction with 4NQO is an excellent tool for studying the progressive changes that occurs during carcinogenesis. Progressive basal lamina degradation and collagen remodeling in connective tissue can be easily analyzed by histochemical analysis with the PAS method as well as with collagen IV immunohistochemistry. Decrease in collagen IV immunoreactivity has been considered as a marker of cancer progression [10, 11, 22]. Multiple studies have shown a discontinuity of collagen IV, at the subepithelial basal lamina during the process of tissue invasion when analyzed by immunohistochemistry [11]. Contrarily, in benign tumors or dysplasia, collagen IV immunoreactivity do not change [9]. In our model, control samples as well as mild dysplasia samples shows similar immunoreactivity, in which an intact basal lamina can be observed. Destruction of the basal lamina became evident in moderate dysplasia by immunohistochemistry. PAS histochemistry is a less sensitive method, since only in severe dysplasia/carcinoma *in situ* changes in the basal lamina can be detected. Collagen IV immunodetection in OSCC is particularly useful, since in head and neck carcinomas the loose of collagen IV immunoreactivity is an early event during carcinogenesis [11]. The most obvious explanation for the decrease in collagen IV immunoreactivity corresponds to degradation, a fact that is supported by the decrease in PAS reactivity. However, it is possible that collagen IV may alter its conformation, which could not be detectable by the antibody. A conformational change of collagen IV could help the adhesion of tumor cells to the basal lamina and thus facilitate tumor invasion. Early detection of abnormalities of the basal lamina may predict the biological behavior of the lesion, which has important therapeutic implications.

Another barrier to the invasive cancer cells is the connective tissue, specially the ECM. As described above, tumor cells induce changes in host cells and ECM. Additionally, host tissues respond against the invading cells. The interplay between invading cancer cells and host response determines the progression of cancer [28]. During 4NQO-induced carcinogenesis, changes in collagen composition is clearly

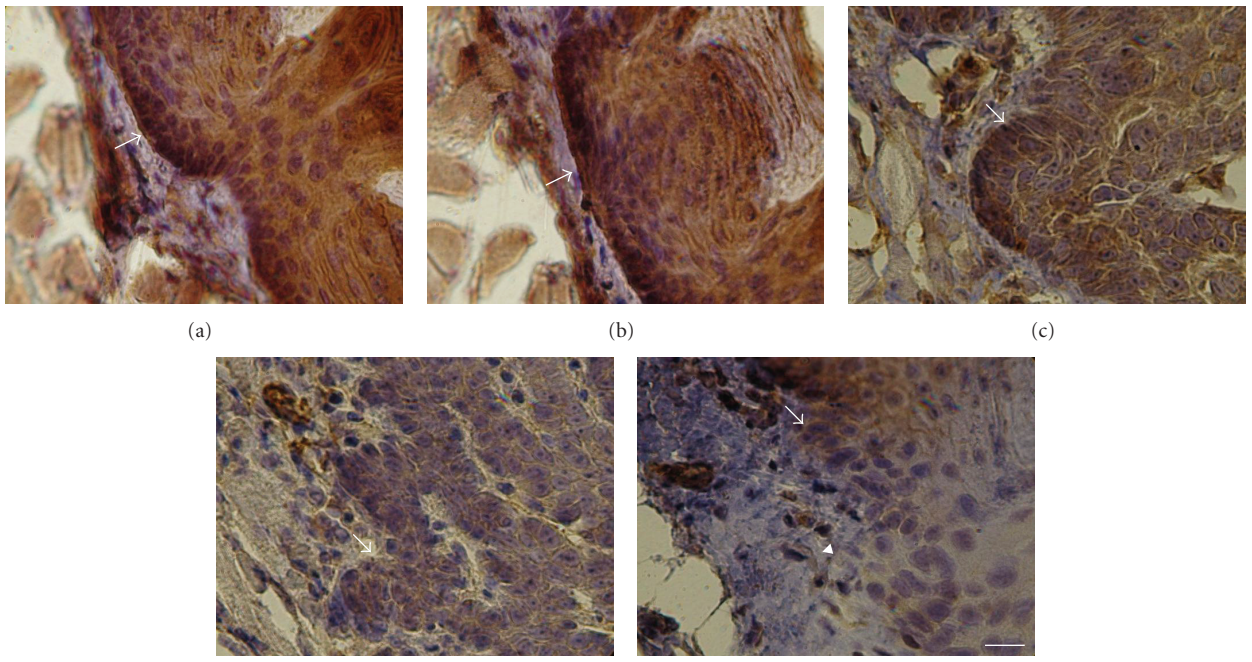

Figure 2: During 4NQO carcinogenesis basal lamina between epithelia and connective tissue is progressively destroyed (collagen IV immunohistochemistry): Histological sections of control, healthy tongue (a), mild (b), moderate (c), and severe (d) dysplasia as well as OSCC (e) were immunostained for collagen IV (arrows). Control and mild dysplasia samples show a strong immunoreactivity for collagen IV, which is weaker in moderate and severe dysplasia and patchy or absent in OSCC, particularly in areas of connective tissue invasion (arrowhead). Bar scale: 10 μm.

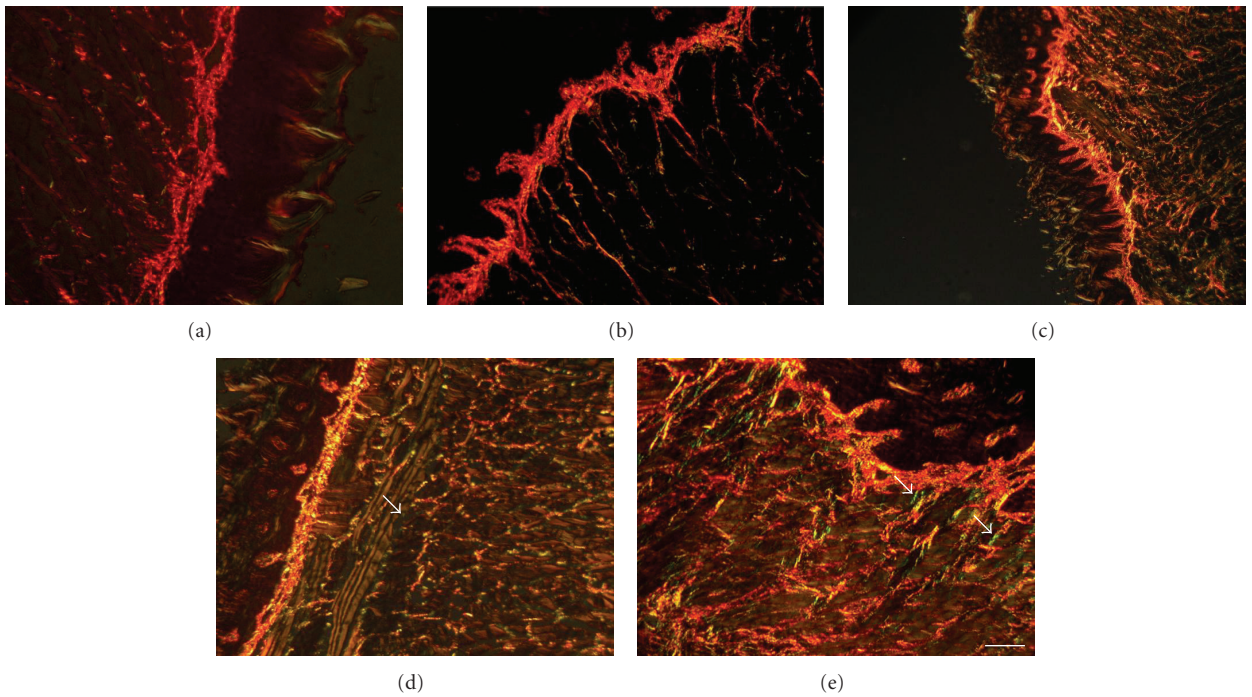

Figure 3: During 4NQO carcinogenesis the ECM connective tissue is progressively remodeled: Histological sections of control, healthy tongue (a), mild (b), moderate (c), and severe (d) dysplasia as well as OSCC (e) were stained for picro sirius red histochemistry. All the samples show presence of collagen I fibers. Interestingly, in severe dysplasia and OSCC collagen III fibers can be observed (arrows). Bar scale: 50 μm.

evident. Collagen I is decreased and replaced by thinner collagen III (Figure 3), changes in collagen fiber composition represents a major disorganization of the "basic skeleton" of ECM. These changes may facilitate the mobilization of the tumor cells inside the connective tissue and increase the probability to reach capillaries and therefore that of metastasis. Other studies have seen similar changes, noting an irregular collagen arrangement and a loosely organized fashion in the periphery of tumor, as well as very thin fibers compared to an area of normal tissue [28].

Our results confirm previous studies that show alterations in ECM in malignant lesions, validating the experimental carcinogenesis induced by 4NQO, since in this model mimics many features and molecular events observed human OSCC development [15]. Particularly, changes in ECM either in basal lamina or in collagen of the connective tissue are evidenced in early in preneoplastic lesions. These changes can be easily evaluated by routine histochemistry or immunohistochemistry and used for diagnosis and prognosis of OSCC.

Acknowledgments

This study was supported by Grants 11080166 and 1120230 (to U. Kemmerling) from FONDECYT and CONICYT-PBCT Anillo ACT 112, Chile.

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