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Expression of Cell Cycle Regulator p16 is not Affected by Diabetes During Oral Oncogenesis

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Abstract. *Background: The tumor suppressor protein p16 plays a vital role in the regulation of the cell cycle. The expression of p16 was investigated in an experimental model of chemically induced carcinogenesis in normal and diabetic (type I) Sprague-Dawley rats. Materials and Methods: Tissue sections ranging from normal oral mucosa to moderately differentiated oral squamous cell carcinoma (OSCC) were studied immunohistochemically. Results: In normal rats p16 expression increased gradually during oral oncogenesis, but a significant increase was observed only in moderately differentiated OSCC ($p=0.038$). On the contrary, in diabetic rats the detected gradual increase was significant in hyperplasia, dysplasia, early invasion and well-differentiated OSCC ($p<0.001$). Nevertheless, there was no significant difference in p16 expression during oral oncogenesis between normal and diabetic animals. Conclusion: It seems that the expression of cell cycle regulator p16 is not affected by diabetes in the studied animal model of oral oncogenesis.*

Oral cancer is one of the most common human neoplasias accounting for 3% of all newly diagnosed cases (1, 2). More than 90% of oral cancer are oral squamous cell carcinomas (OSCC) (3, 4). Late presentation, lack of suitable markers for early detection and failure of advanced lesions to respond to chemotherapy contribute to the poor outcome of these types of cancer. A variety of molecular factors are involved in malignant transformation, including alterations in oncogenes and tumor suppressor genes that result in destabilization of several growth control systems (5). These events are further influenced by exposure to environmental agents, including smoking, heavy alcohol consumption, and certain viruses (*e.g.* types of human papillomavirus) (6, 7).

Deregulation of cell proliferation is considered to be a step of vital importance in cancer evolution. Therefore, the definition of key events that underlie the control of this mechanism is likely to contribute to our understanding of cancer development and progression. Orderly progression of cells through the cell cycle is orchestrated by sequential interaction and activation of cyclin-dependent kinases (cdks) existing in complexes with their cyclin substrate(s) (8). Different cdks and cyclin-subunits are required in each phase of the cell cycle (9). Though there may be a number of different protein targets for these cyclin/cdk activities required for driving the cell from the G1- into the S-phase, the retinoblastoma gene product (pRb) is certainly a major substrate (10).

Inhibitory proteins that react with cyclin/cdk complexes and down-regulate their activity have been identified recently. The protein product of the *CDKN2/INK4a* gene, p16, inhibits the cell cycle directly by forming binary complexes with the cyclin D/cdk4 complex (11). Previous studies have shown that the pathway involving Ras/mitogen-activated protein kinase (MAPK) signalling induces production of p16 (12). Ras/MAPKs phosphorylate members of the ternary complex factor (TCF) family which leads to the transcription of immediate early genes such as *JunB* (13, 14). JunB, a member of the Jun family, has been reported to directly target and activate p16 (15). Loss of the p16 gene may lead to overexpression of cyclin D *via* the Ras/MAPK cascade which eventually results in increased cell proliferation and transformation (16).

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The *CDKN2* gene has been mapped to human chromosome 9p21, a region frequently deleted in many types of cancer, suggesting that it is a candidate tumor suppressor gene. However, the frequency of *CDKN2*/*p16* genetic alterations in primary tumors appears to be much lower than that in tumor cell lines, leading some authors to argue that the loss of *p16* may represent a tissue culture adaptation artifact (17, 18). However, recent studies of lung cancer, head and neck cancer, and glioma indicated that the inactivation of the *CDKN2*/*p16* gene is more frequently induced by methylation of the promoter region than by alteration of the coding sequence (19-21). In an animal model (hamster) of oral carcinogenesis, we observed that inactivation of p16 occurs early at the premalignant stage of mucosal dysplasia (22).

It is well established that diabetes mellitus is correlated with a variety of oral conditions (23). Several metabolic and immunological changes which affect oral mucosa and are associated with a variety of oral conditions can be attributed to diabetes mellitus (24). To our knowledge, there are rare data in the literature concerning p16 expression in diabetes. One study has suggested that circulating progenitor blood cells from patients with diabetes mellitus display reduced proliferative capacity and up-regulation of p16 due to hyperglycemia (25). In accordance, another study discovered increased levels of p16 in diabetic rats compared to normal rats in glomerular cells (26).

In light of the above-mentioned up-regulation of p16 in diabetes and our recent finding of a molecular association between oral cancer and diabetes in an animal model (27), we decided to study the effect of diabetes on p16 involvement in oral oncogenesis. Therefore, using the same experimental animal model of diabetic (type I) and normal rats with induced OSCC, we examined the expression of p16 in normal oral mucosa tissues and in sequential stages of tumor formation.

Materials and Methods

Thirty-seven female Sprague-Dawley rats purchased from the Hellenic Pasteur Institute (Athens) at the age of six weeks, weighing approximately 135 g each, were used in this study. The rats were handled in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (28). The animals were randomly divided into four groups. A) Group D (n=6): Diabetic rats without carcinogenesis, B) Group Dc (n=13): Diabetic rats used for induced carcinogenesis, C) Group N (n=6): Normal rats without carcinogenesis, D) Group Nc (n=12): Normal rats used for induced carcinogenesis.

The induction of diabetes was performed in 19 previously overnight fasted animals by a single intraperitoneal injection of streptozotocin (STZ) dissolved in saline buffer at a dose of 70 mg/kg of weight (ZANOSAR, Pharmacia & Upjohn Co., USA) and determined by glucose levels in blood after three weeks, as described elsewhere (27). Oral cancer was induced in Dc and Nc animals by application of the carcinogen 4-nitroquinoline *N*-oxide (4NQO) at a concentration of 5% in propylene glycol 3 times per week for 5 months in the rats' hard palate (Fluca AG Chemische Fabrik, Switzerland), as described elsewhere (27). Clinical signs of oral lesions putatively tumor-related were observed within 6 months after the last application of carcinogen. After sacrifice of animals by ether treatment, the oral regions with cancer (mainly palate and tongue) of Dc and Nc rats and the respective regions of D and N rats were excised for immunohistochemical analysis (27).

Table I. *Histological status of biopsies in groups N, D, Nc and Dc.*

	Number of rats			
	Control groups		Experimental groups	
	(N)	(D)	(Nc)	(Dc)
Normal tissue	3	5		
Hyperplasia	3	1		1
Dysplasia			1	4
Early invasion			1	1
Well-differentiated carcinoma			6	4
Moderately-differentiated carcinoma			4	3

N: normal rats without carcinogenesis; D: diabetic rats without carcinogenesis; Nc: normal rats with induced carcinogenesis; Dc: diabetic rats with induced carcinogenesis.

Pathological evaluation. The histological status of the lesions was defined after examination of the complete section under light microscopy and the tissue profiles were classified into the following categories: normal mucosa, hyperplasia, dysplasia, early invasion, well- and moderately differentiated carcinoma. In every sample all possible different lesions were evaluated.

Immunohistochemical analysis. Sections were prepared from each specimen and were used for immunohistochemical detection of p16 with monoclonal primary antibody against p16 (F-12:sc-1661, 1:500 dilution; Santa Cruz Biotechnology Inc, CA, USA) as described elsewhere (29). Tonsil tissue which strongly expresses p16 was used as positive control. Negative controls for each biopsy were processed in the same manner, using phosphate-buffered saline (PBS) instead of the primary antibody. All slides were independently reviewed by two investigators blindly. The consecutive hematoxylin-eosin-stained slides were evaluated by a pathologist experienced in oral pathology, without knowing the p16 staining patterns. Results of p16 expression were presented as the percentage of positively stained cells in each sample.

Statistical analysis. For each animal, the percentage of p16 expression in all possible lesions was evaluated. All lesions were accumulated and classified into the following categories: normal mucosa, hyperplasia, dysplasia, early invasion, well- and moderately differentiated carcinoma. Statistical analyses were performed using the two-tailed Student's *t*-test for each group of animals and each histological category. The percentage of positively stained cells

Table II. *Percentage of p16-positive cells in the various tissue categories for normal (N, Nc) and diabetic (D, Dc) rats.*

	Non-cancerous and precancerous				Tumor	
	Normal oral mucosa	Oral mucosal hyperplasia	Oral mucosal dysplasia	Early invasion	OSCC	
					Well-differentiated	Moderately differentiated
Normal rats						
Number of tissues	4	9	4	4	6	4
Mean percentage	2.75	3.33	5.5	5	12.5	12.5
Mean non-cancerous and precancerous condition		4				
Probability of *t*-test		N.S.	N.S.	N.S.	N.S.	$p=0.038$
Diabetic rats						
Number of tissues	22	33	21	4	23	11
Mean percentage	2.36	4.51	5.14	10	8.48	5.64
Mean non-cancerous and precancerous condition		4.75				
Probability of *t*-test		$p=0.002$	$p=0.0001$	$p<0.0001$	$p<0.0001$	N.S.

from each non cancerous or precancerous condition (hyperplasia, dysplasia) were compared to normal tissue, while the percentage of positively stained cells from each tumor (early invasion, well- and moderately differentiated carcinoma) were compared to the mean value of percentages of the non-cancerous and precancerous conditions. In all cases with no normal distribution, the results of both the Wilcoxon test and two-tailed Student's *t*-test provided the same level of significance.

Results

Table I summarizes the histological status of biopsies examined (normal mucosa, hyperplasia, dysplasia, early invasion, well- and moderately differentiated carcinoma) both from normal rats (N, Nc) and diabetic rats (D, Dc). A progression towards OSCC formation with increased time of carcinogen application is evident.

The percentages of p16-positive cells in the various tissue categories for normal animals (groups N and Nc) and diabetic animals (groups D and Dc) are shown in Table II. Expression of p16 was detected in the nuclei of neoplastic and non-neoplastic cells in all cases, with the percentage of positive cells ranging from 1% to 25% of the total cell population (Figure 1). Most cases also displayed, at least focally, cytoplasmic reactivity, which was disregarded as non-specific staining. Reactivity was unusually strong with a granular or diffuse quality.

In normal rats, a statistically significant higher p16 expression ($p=0.038$) was only found in moderately differentiated OSCC when compared to the mean percentage of the non-cancerous and precancerous conditions (Table II). On the contrary, in diabetic rats (Table II) statistically significant differences were found in the categories of oral mucosal hyperplasia ($p=0.002$) and oral mucosal dysplasia ($p=0.0001$) when compared to normal oral mucosa. In addition, statistically significant differences were observed in early invasion ($p<0.0001$) and well-differentiated carcinoma ($p<0.0001$) when compared to the mean percentages of the non-cancerous and precancerous conditions (Table II).

Finally, there was no significant difference between normal and diabetic animals in expression of p16 in the various stages of oral oncogenesis (data not shown). Figure 2 depicts the pattern of p16 expression in both normal and diabetic rats. It seems that p16 expression gradually increases during oncogenesis in normal rats. Nevertheless, in diabetic animals, p16 follows a similar pattern of gradual increased expression except for the stage of moderately differentiated OSCC, where a non-significant decrease in p16 protein levels is evident. Despite the apparent difference in p16 expression between normal and diabetic rats, especially in moderately differentiated OSCC, the statistical analysis did not reveal any significant data, probably due to the small number of representative histological lesions analyzed.

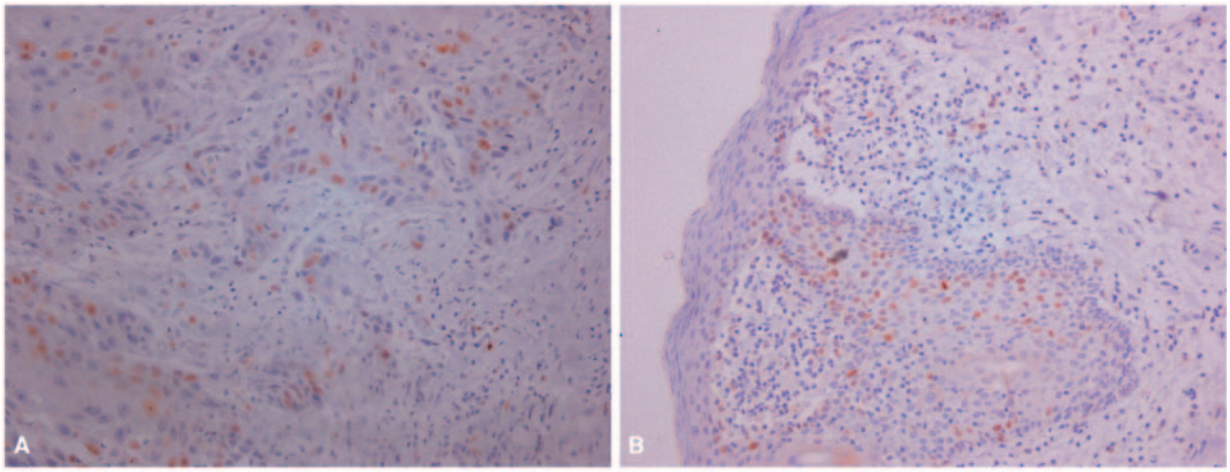

Figure 1. *(A) Increased p16 nuclear immunoreaction in a moderately differentiated invasive carcinoma from a normal rat (x200); (B) Hyperplastic oral mucosa of a diabetic rat with increased p16 immunoreactivity.*

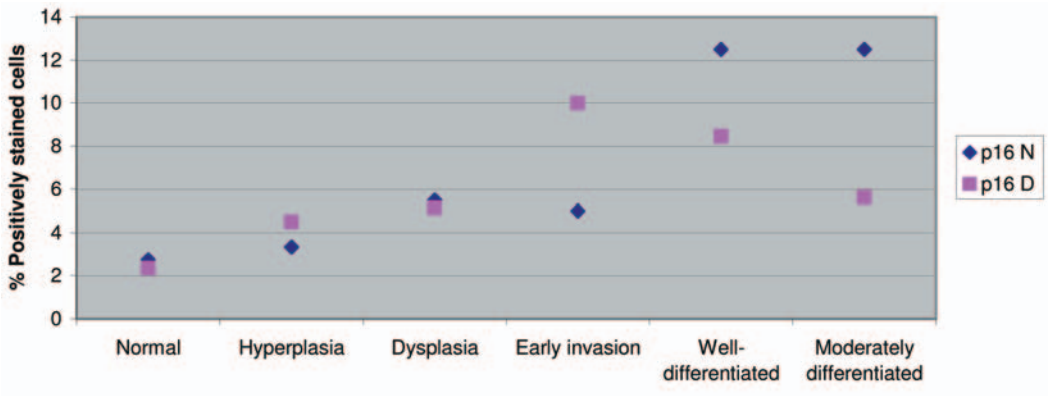

Figure 2. *Expression of p16 in the different tissue categories in normal (N) and diabetic (D) rats. The values represent the mean percentage of positively stained cells in each histological category.*

Discussion

The expression of p16 was investigated in sequential stages of oral oncogenesis, varying from normal oral mucosa to moderately differentiated oral squamous cell carcinomas in biopsies from both normal and diabetic rats, with or without induced oral cancer. In normal rats, p16 expression increased gradually during oral oncogenesis. In diabetic animals, p16 also increased progressively during oral oncogenesis except for the stage of moderately differentiated OSCC, where a slight decrease in p16 protein levels was evident. Nevertheless, the comparison between normal and diabetic animals revealed no statistical differences, indicating that diabetes does not appear to affect the expression of p16 during oral oncogenesis.

The present study is, to our knowledge, the first investigating the expression of the cell cycle regulator p16 in an experimental animal system of chemically induced oral carcinogenesis in normal and diabetic rats. A previous similar experimental study by our group implicated diabetes mellitus as a risk factor for the development of OSCC (27). In accordance with our previous findings, some epidemiological studies have associated diabetes with oral precancerous and cancerous lesions (30-32).

There are several reports regarding the expression of p16 and other cell cycle proteins in human OSCC suggesting alterations of the p16 pathway at an early stage of oral oncogenesis, most commonly in the precancerous stage of dysplasia (22, 33-36). Furthermore, other studies have suggested a progressive tendency for overexpression of p16 from normal tissue to precancerous tissues and then to OSCCs, in accordance with the findings of the present animal model (37, 38).

A possible explanation for the increased amount of p16 protein during oral oncogenesis observed in the present study may be the unscheduled transactivation of the *p16* gene. Increased accumulation of p16 mRNA and protein has been reported in response to cellular senescence, oncogenic hyperactivity of Ras and inactivation of Rb (39). Previous studies by our group have revealed progressive increase of N-ras expression during oral oncogenesis in both normal and diabetic animals (40). Oncogenic hyperactivity of ras which was evident in the previous studies may result in increased expression of MAP kinases leading ultimately to the transcriptional activation of p16.

Alternatively, CDK4 overexpression, possibly induced by extrinsic or intrinsic factors, may decrease functional phosphorylation of Rb protein, leading to increased p16 expression in a compensatory manner. This in turn may result in weak feedback inhibition of Rb, thus maintaining the cell cycle in normal order (37). Previous studies in oral premalignant lesions have detected a strong reciprocal relationship between Rb and p16 expression (36, 41). Since the *p16* gene is transcriptionally repressed by Rb protein, p16 mRNA and its encoded protein accumulate to high levels in cells lacking Rb (42). Interestingly, one study in a rat model of NQO-induced tongue oncogenesis revealed a decrease of Rb expression in premalignant stages followed by almost an absence of expression in tumor stages (43).

It is known that in diabetes the oxidation equilibrium breaks down. The elevated glucose concentration, excessive formation of free radicals, and protein glycation depress the activities of antioxidant scavengers and enzymes. These noxious processes may cause serious damage to the biological structures at the molecular level. The inducing role of free radicals and oxidative stress in carcinogenesis is a well-known fact (44). There are rare data in the literature concerning p16 expression in regard to diabetic status. One study has suggested that circulating progenitor cells from patients with diabetes mellitus display reduced proliferative capacity and up-regulation of p16 due to hyperglycemia (25). In addition, another study discovered increased levels of p16 in glomerular cells in diabetic rats compared to normal rats (26). Nevertheless, in the present study, the comparison between normal and diabetic animals revealed no statistical differences, indicating that diabetes does not influence the expression of p16 tumor suppressor protein during oral oncogenesis.

Conclusion

Our study did not reveal any significant effect of diabetes on the p16 cell cycle regulatory pathway. Therefore, it seems that p16 may not be implicated in the diabetes-related oncogenesis in the oral region of the present experimental animal system.

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