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Diabetes does not Influence Oral Oncogenesis Through Fibroblast Growth Factor Receptors

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Abstract. *Background: Increased expression of fibroblast growth factors and their receptors (FGFRs) has recently been described in oral squamous cell carcinoma. In addition, we have previously described a molecular basis for an association between oral cancer and diabetes. The expression of FGFR-2 and FGFR-3 investigated in an experimental model of chemically induced carcinogenesis in normal and diabetic (type I) rats. Materials and Methods: Tissue sections ranging from normal mucosa to moderately-differentiated oral squamous cell carcinoma were studied using monoclonal antibodies against FGFR-2 and FGFR-3 proteins. Results: A similar pattern of elevated FGFR-2 and FGFR-3 expression was observed in the initial stages of oncogenesis for both diabetic and non-diabetic animals. In the last stages of oral oncogenesis, the expression of both proteins remained relatively stable. Conclusion: It seems that diabetes does not affect the FGFR-2 and FGFR-3 pattern of expression throughout the various stages of oral oncogenesis.*

The majority of malignant tumours of the oral cavity are oral squamous cell carcinomas (OSCC), followed by adenocarcinomas and other rare types of malignant tumours (1). It is generally accepted that oral carcinogenesis is a multi-step process of accumulated genetic damage including the overexpression of oncogenes, the inactivation of tumour suppressor genes and the alteration of genes involved in the metabolism of carcinogens or DNA repair (2). The search for other factors involved in oral carcinogenesis is important in order to increase our understanding of this process.

Traditionally, diabetes mellitus has been correlated with a variety of inflammatory oral lesions, but only recently have epidemiological studies incriminated it as a risk factor for the development of OSCC, as well as oral premalignant lesions, such as leukoplakia (3-5). To our knowledge, the first study investigating a molecular basis for the association between oral cancer and diabetes was published by our group (6). Using an animal model, we found that a possible mechanism linked to both diseases involves insulin receptor substrate-1 (IRS-1) and focal adhesion kinase pp125 (6). Insulin deficiency results in reduced amounts of insulin receptor and, consequently, in cytoskeleton changes and reduced cell adhesion, an initial step towards neoplasia (6).

There are additional growth factors the expression of which is altered in diabetic tissues compared to normal ones. These include fibroblast growth factors (FGFs), a family of related polypeptides which exert their effects through the transmembrane high-affinity fibroblast growth factor receptors (FGFRs), including FGFR-2/FGFR-3 and, in this way, regulate cell proliferation, differentiation, and function in a number of tissue processes, including normal development, carcinogenesis and metastasis (7-9).

Recently, increased expression of fibroblast growth factors and their receptors has been described in squamous cell carcinoma of oral mucosa, suggesting that these growth factors and subsequently their receptors may contribute to cancer cell growth (10-13). The expression of FGFs was found altered in diabetic tissues compared to normal ones in various tissues including human placenta, skin and

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periodontal ligament cells (14-16). Taken this into consideration, we investigated the expression of FGFR-2 and FGFR-3 in an experimental model of induced OSCC in diabetic (type I) and non-diabetic rats in normal oral mucosa tissues and in various tissues exhibiting both precancerous and cancerous lesions.

Materials and Methods

Animals. Thirty-seven female Sprague-Dawley rats were used in this study. They were purchased from the Hellenic Pasteur Institute (Athens, Greece) at the age of six weeks and weighing approximately 135 g each. 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 (NIH Publication No. 85-23, revised 1996).

Induction of diabetes. Diabetes was induced in nineteen rats by a single intraperitoneal injection of streptozotocin (STZ) dissolved in saline buffer at a dose of 70 mg/kg body weight (ZANOSAR, Pharmacia & Upjohn Co., USA). The animals were fasted overnight prior to STZ administration. Three weeks after STZ injection, blood glucose levels were determined with a strip-operated sensor in blood samples obtained by tail prick. Glucose levels were >240 mg/dl in 12 of 22 STZ-injected animals which were considered to be diabetic.

Experimental carcinogenesis. The Sprague-Dawley rats used in this study were randomly divided into the following groups: i) Group D (n=6): diabetic rats without carcinogenesis; ii) Group Dc (n=13): diabetic rats used for induced carcinogenesis; iii) Group N (n=6): normal rats without carcinogenesis; iv) Group Nc (n=12): normal rats used for induced carcinogenesis.

Induction of carcinogenesis was accomplished by the application of 5% of the carcinogen 4-nitroquinoline *N*-oxide (4NQO) dissolved in propylene glycol to the hard palate of Dc and Nc animals (*Fluka AG Chemische Fabrik, Buchs, Switzerland*). Furthermore, in the hard palate of animals from group D and N, propylene glycol solution alone was applied.

The applications of the carcinogen started three weeks after the induction of diabetes and was performed 3 times per week for 5 months. The intraoral and general condition of each rat were inspected every week and body weight was registered once a month during that period of 5 months. Once oral cancer clinical signs were observed in both Dc and Nc animals (2-6 months after last application of carcinogen), they were sacrificed by ether treatment. The oral cancerous regions (mainly palate and tongue, as well as in some cases maxilla and cheek) were excised for immunohistochemical analysis. Rats of groups D and N were sacrificed following the sacrifice of all animals from groups Dc and Nc.

The histological status of the lesions was defined and the tissue profiles were classified into the following categories: normal mucosa, hyperplasia, dysplasia, early invasion, well- and moderately differentiated carcinoma. The percentages of positive expression for both FGFR-2 and FGFR-3 were counted in several regions of each specimen which contained more than one histological lesion. Sections were prepared from each specimen and were used for immunohistochemical detection of FGFR-2 and FGFR-3 proteins with monoclonal primary antibodies against FGFR-2 (Bek C-17: sc-122, Santa Cruz Biotechnology, Inc., diluted 1:100) and FGFR-3 (FGFR-3 C-15: sc-123, Santa Cruz Biotechnology, Inc., diluted 1:200) as described elsewhere (17).

Statistical analyses were performed using the two-tailed Student's *t*-test for each group of animals and each histological category. The percentages of positively stained cells from each non-cancerous or precancerous condition (hyperplasia, dysplasia) were compared to normal tissue, while the percentages of positively-stained cells from each tumor (early invasion, well- and moderately differentiated carcinoma) were compared to the mean percentage of the 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.

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 (n=6): normal rats without carcinogenesis, D (n=6): diabetic rats without carcinogenesis, Nc (n=12): normal rats used for induced carcinogenesis, Dc (n=13): diabetic rats used for induced carcinogenesis.

Results

The histological status of biopsies observed in various regions of rats from all groups (N, Nc, D, Dc) after induced oral oncogenesis is summarized in Table I. The experimental model seems valid since several normal, non-cancerous, precancerous and cancerous regions in the various tissue biopsies were collected and further analysis of immunostaining data was implemented (Figures 1, 2).

The percentages of positive expression of FGFR-2 and FGFR-3 in the various histological categories for diabetic animals from groups D and Dc are shown in Table II. FGFR-2 expression was found to be significantly higher in non-cancerous ($p<0.01$) and precancerous stages ($p<0.001$) and in the cancerous stage of early invasion ($p<0.05$). In the remaining stages of oral oncogenesis, a non-statistically significant decrease in FGFR-2 expression was observed. FGFR-3 expression followed a similar pattern of elevated expression in the initial stages of the oncogenic process and decreased expression in the last stage of moderately differentiated OSCC, although not statistically significant.

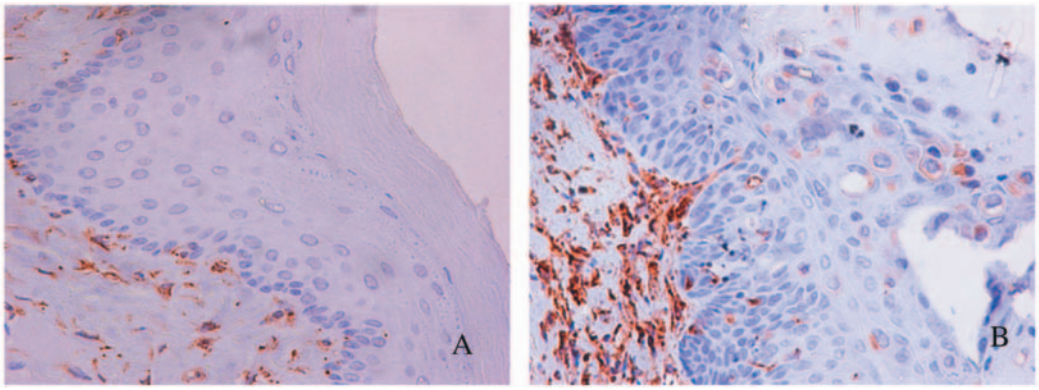

Figure 1. *Immunohistochemical staining of oral tissues using FGFR-2 antibody: (A) FGFR-2 low stromal immunoexpression in a non-dysplastic oral epithelium in a normal rat (x400); (B) strong FGFR-2 stromal immunoexpression in dysplastic oral mucosa in a diabetic rat (x400).*

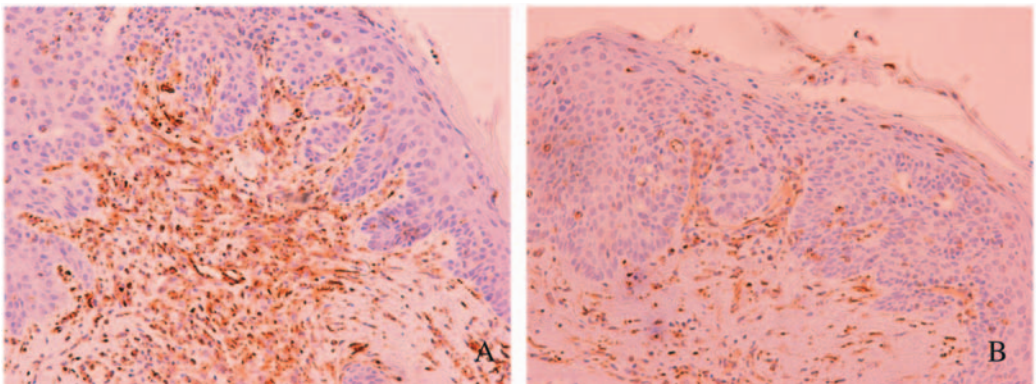

Figure 2. *Immunohistochemical staining of oral tissues using FGFR-3 antibody: (A) FGFR-3 strong stromal immunoexpression in an area of early invasion in a diabetic rat (x400); (B) FGFR-3 stromal immunoexpression in dysplastic oral mucosa (x200) in a normal rat.*

The percentages of positive expression of FGFR-2 and FGFR-3 in the various histological categories for animals from groups N and Nc are shown in Table III. FGFR-2 expression significantly increased up to the stage of dysplasia, remained stable in early invasion, decreased in well differentiated carcinoma and increased in moderately differentiated carcinoma but presented no statistical difference. FGFR-3 presented virtually the same pattern of expression.

There was no significant difference between normal and diabetic animals in expression of FGFR-2 in the various stages of oral oncogenesis (data not shown). Accordingly, the comparison of FGFR-3 expression between normal and diabetic animals in the various stages of oral oncogenesis also failed to reveal any statistically significant difference (data not shown).

Discussion

In this study, the expression of FGFR-2 and FGFR-3 was investigated in several stages of oral oncogenesis, from normal oral mucosa to moderately differentiated oral carcinomas, obtained from normal and diabetic rats with or without induced oral cancer. FGFR-2 and FGFR-3 expression presented a similar pattern of elevated expression in the initial stages of the oncogenesis process for both diabetic and non-diabetic animals (Figure 3). In the last stages of oral oncogenesis, the expression of both proteins remained relatively stable (Figure 3). There was no significant difference between normal and diabetic rats in expression levels of FGFR-2 and FGFR-3 in the various stages of oral oncogenesis.

There is scarce literature concerning FGFR-2 and FGFR-3 expression in oral tissues from diabetic and non-diabetic patients. In one study which investigated the expression of FGFs and their receptors in type I diabetes human placenta, diabetes did not seem to influence their expression compared to normal term tissue (14). In addition, a study investigating the effect of insulin and glucose levels on retinal glial cell activation, suggested that decreased systemic insulin and high glucose levels contribute to decreased FGF-2 production (18). Furthermore, one group investigated the expression of bFGF in gingival fibroblasts and periodontal ligament cells

Table II. *Percentage of FGFR-2 and FGFR-3 positive cells in the various tissue categories for diabetic rats (D and Dc).*

		Non-cancerous and precancerous		Tumour		
	Normal oral mucosa	Oral mucosal hyperplasia	Oral mucosal dysplasia	Early invasion	Well-differentiated OSCC	Moderately-differentiated OSCC
FGFR-2						
Mean percentage	2.59 (N=22)	4.84 (N=33)	12.62 (N=21)	15 (N=4)	10.21 (N=23)	7.72 (N=11)
		7.87				
Probability of *t*-test		<0.01[1]	<0.001[1]	<0.05[1]	N.S.[2]	N.S.[2]
FGFR-3						
Mean percentage	1.72 (N=22)	5.87 (N=33)	10.66(N=21)	7.5 (N=4)	11.17 (N=23)	8.54 (N=11)
		7.74				
Probability of *t*-test		<0.01[1]	<0.001[1]	N.S.[1]	<0.05[2]	N.S.[2]

[1]Compared to normal mucosa; [2]compared to mean precancerous mucosa. N.S.: No statistical difference. N: Number of regions corresponding to each histological category.

Table III. *Percentage of FGFR-2 and FGFR-3 positive cells in the various tissue categories for normal rats (N and Nc).*

		Non-cancerous and precancerous		Tumour		
	Normal oral mucosa	Oral mucosal hyperplasia	Oral mucosal dysplasia	Early invasion	Well-differentiated OSCC	Moderately-differentiated OSCC
FGFR-2						
Mean percentages	1.25 (N=4)	4.55 (N=9)	12.5 (N=4)	12.5 (N=4)	9.83 (N=6)	11.25 (N=4)
		7				
Probability of *t*-test		<0.05[1]	<0.001[1]	<0.05[1]	N.S.[2]	N.S.[2]
FGFR-3						
Mean percentages	3 (N=4)	6 (N=9)	11.25 (N=4)	12.5 (N=4)	7.8 (N=6)	12.5 (N=4)
		7.61				
Probability of *t*-test		N.S.[1]	<0.05[1]	N.S.[1]	N.S.[2]	N.S.[2]

[1]Compared to normal mucosa; [2]compared to mean precancerous mucosa. N.S.: No statistical difference. N: Number of regions corresponding to each histological category.

from diabetics and non-diabetics and suggested that fibroblasts exhibiting the greatest increase in growth in response to high glucose also exhibited increased expression of bFGF (15). Nevertheless, our findings indicate that diabetes does not affect FGFR-2 and FGFR-3 pattern of expression throughout oral oncogenesis.

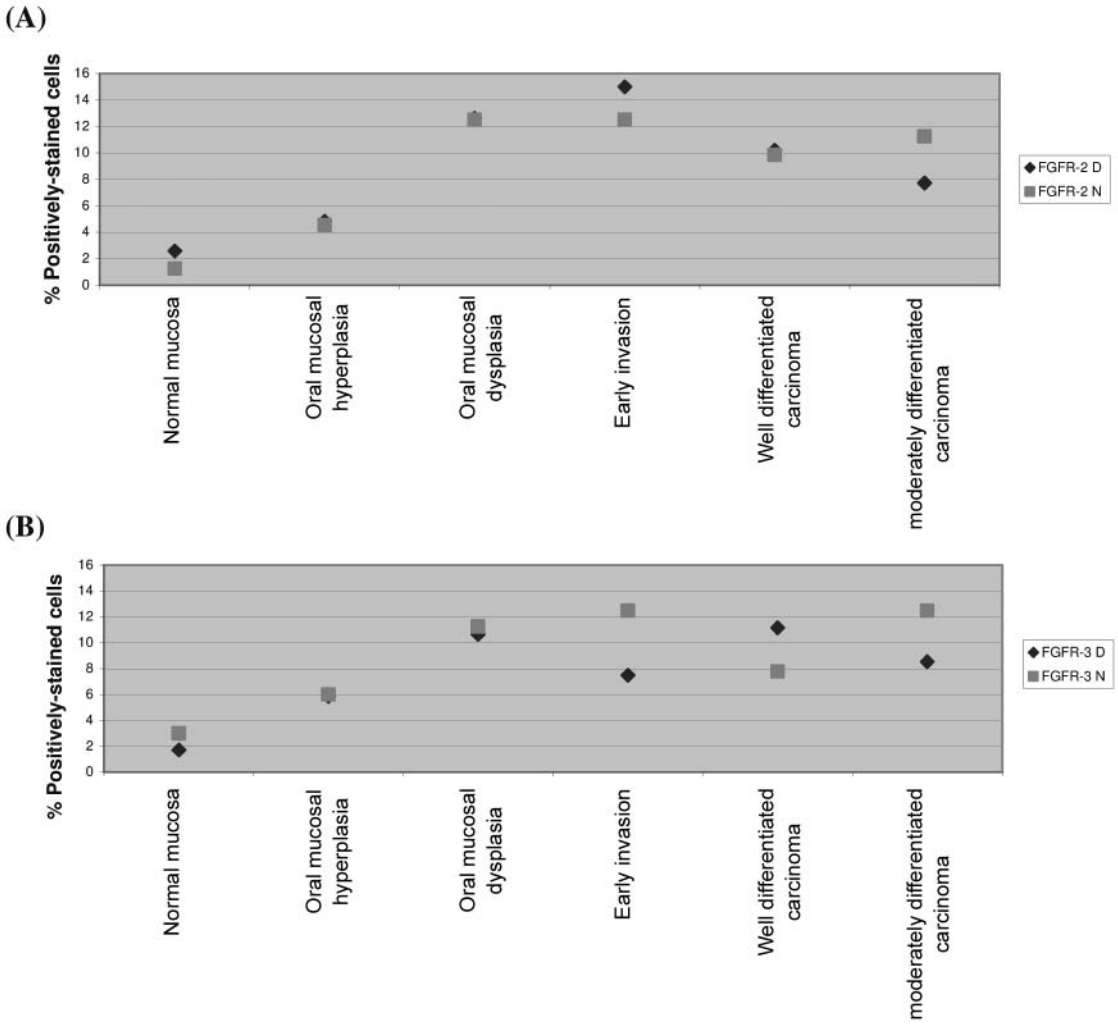

Figure 3. *Expression of (A) FGFR-2 and (B) FGFR-3 in the different tissue categories in normal (N) and diabetic (D) rats.*

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