Abstract. Oral squamous cell carcinoma (OSCC) is the sixth most common malignancy in humans including type I diabetic and normal rats. Tobacco and alcohol, as well as dysregulation of oncogenes and tumor suppressor genes, epigenetic changes and mitochondrial mutations have been implicated in OSCC development. Recent epidemiological studies have incriminated diabetes mellitus as a risk factor for the development of OSCC, as well as oral premalignant lesions. Recently, an animal model was employed to study the influence of diabetes on signal transduction pathways in every stage of oral cancer development, from normal mucosa to hyperplasia, dysplasia, early invasion, well differentiated OSCC and moderately differentiated OSCC. Diabetes was induced by streptozotocin and chemical carcinogenesis was induced by the carcinogen 4-nitroquinoline N-oxide. The expression of EGFR, erbB2, erbB3, FGFR-2, FGFR-3, c-myc, N-ras, ets-1, H-ras, c-fos and c-jun, the tumor suppressor genes p53 and p16, apoptosis markers Bax and Bcl-2, and the cell proliferation marker Ki-67 in the sequential stages of rat oral oncogenesis was investigated. Diabetes seems to promote the activation of the Ras/Raf/MAPK signal transduction pathway mainly by induction of erbB2 and erbB3 receptors, leading to increased cell proliferation, while there was no difference in apoptosis levels during oncogenesis.

Animal Models of Oral Squamous Cell Carcinoma

Oral squamous cell carcinoma (OSCC) is the sixth most common malignancy and constitutes a major health problem which is associated with severe morbidity and <50% long-term survival, despite advances in treatment with surgery, radiation and chemotherapy (1, 2). More than 300,000 new cases of OSCC are being diagnosed each year worldwide, but the poor prognosis of oral cancer has not improved significantly over the last four decades (1, 3). The most important risk factors for the development of oral cancer are tobacco and alcohol (4). In addition, dysregulation of oncogenes and tumor suppressor genes, cytogenetic changes, epigenetic changes and mitochondrial mutations have been implicated in OSCC development (5). Therefore, understanding the molecular mechanisms involved in the initiation and progression to malignancy of oral cancer will help to improve its prognosis and in the development of new forms of treatment.

Due to the immediate need for the development of several early diagnostic and prognostic markers of oral cancer, the sequential alterations in molecular mechanisms of OSCC development must be established (6). Oral oncogenesis is a multi-step process which involves various histological changes such as hyperplasia, dysplasia and the development of carcinomas. In humans, the molecular analysis of these multiple steps is hampered by the unavailability of biopsies from all stages of carcinogenesis (6). Therefore, animal models for OSCC development have been generated (including hamster, rat and mouse models) which allow the reproducible pathological characterization of all stages and their comparison with normal tissue (7, 8).

The OSCC induction in animals was first demonstrated in a hamster cheek pouch with the aid of polycyclic aromatic hydrocarbons such as 9,10-dimethyl-1,2-benzanthracene (DMBA), 20 methylcholanthrene
(20MC) and 3,4-benzopyrene (3,4BP) (9). In the early 1970s, the rat model of oral carcinogenesis was introduced, widely using 4-nitroquinoline-1-oxide (4NQO), a synthetic water-soluble carcinogen, to study various stages of oral oncogenesis (10-12). 4NQO exerts potent intracellular stress and its metabolic product binds to DNA predominantly at guanine residues (6). The rat model of 4NQO carcinogenesis produces a spectrum of preneoplastic and neoplastic lesions and, since it parallels OSCC development in humans, it is considered as the preferred model for the study of OSCC (13). Several markers which have the potential to indicate and predict the evolvement of an invasive malignant neoplasm following exposure to 4NQO have been investigated, such as tumor suppressor protein p53, E- and P-cadherin, proliferating markers Proliferating cell nuclear antigen (PCNA) and Bromodeoxyuridine (BrdU), Argyrophilic nucleolar organizer region (AgNOR) parameters, cytokeratin profile, and the nuclear profile of basal epithelial cells (14-20). In addition, the apoptosis-related proteins Bcl-2 and Bax have been shown to be altered in the rat 4NQO model of OSCC (21).

Traditionally, diabetes is correlated with a variety of oral conditions, such as periodontal disease, dental caries, stomatitis, glossitis and sensory changes (22, 23). Nevertheless, it was not considered to be involved in the pathogenesis of oral cancer, although correlations between diabetes and inflammatory oral lesions were established as early as the 19th century (24). Interestingly, recent epidemiological studies have incriminated diabetes mellitus as a risk factor not only for the development of OSCC, but also for oral premalignant lesions such as leukoplakia (25, 26). Type I diabetes, formerly known as either juvenile-onset diabetes (because of the early age of onset) or insulin-dependent diabetes mellitus (because of the clinical need for insulin), is now widely thought to be an organ-specific autoimmune disease resulting from destruction of insulin-producing pancreatic β-cells (27). Insulin deficiency results in reduced amounts of insulin receptor and consequently in cytoskeleton changes and reduced cell adhesion. In a previous study by our group, the molecular basis for the putative association between oral cancer and diabetes and the possible involvement of insulin receptor substrate-1 (IRS-1) and focal adhesion kinase (FAK) was investigated in diabetic (type I) and normal rats with induced oral squamous cell carcinoma (28). Our findings suggested that the IRS-1/FAK pathway is altered by diabetes resulting in reduced cell adhesion and possibly increasing the risk for oral cancer (28). Interestingly, type I diabetic patients not insulin-regulated might have a greater risk for developing oral cancer than do the general population.

The Rat Model of Sequential Oral Oncogenesis

In order to study the molecular pathways involved in the sequential stages of OSCC development and the association between diabetes and oral cancer, an experimental model of OSCC induced in diabetic (type I) and normal rats was used. A brief description of the performed experimental procedures follows, since they have been described in detail elsewhere (28).

**Induction of diabetes and experimental carcinogenesis**. Thirty-seven female Sprague-Dawley rats purchased from the Hellenic Pasteur Institute (Athens) at the age of six weeks and 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 (NIH Publication No. 85-23, revised 1996). 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 confirmed by glucose levels in the blood after three weeks, as previously described (28).

Oral cancer was induced in Dc and Nc animals by application of the carcinogen 4NQO at a concentration of 5% in propylene glycol 3 times per week for 5 months in the rats’ hard palate (Fluka AG Chemische Fabrik, Switzerland), as described elsewhere (28). Clinical signs of oral lesions putatively tumor-related were observed within 6 months after 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 (28).

**Pathological and immunohistochemical 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, a number of all possible representative histological lesions were analysed. The biopsies from the 37 animals were fixed in 10% neutralized formaldehyde solution and embedded in paraffin. Sections 4 μm-thick were prepared from each specimen and were mounted on Super Frost Plus-coated glass slides (Menzel and Co., Braunschweig, Germany). All sections were used for
Detection of Bax, Bcl-2, Ki-67, p53, c-myc, N-ras, H-ras, c-fos, c-jun, epidermal growth factor receptor (EGFR), erbB2, erbB3, fibroblast growth factor receptor-2 (FGFR-2), fibroblast growth factor receptor-3 (FGFR-3) and p16 proteins, using a standard immunohistochemical technique (DAB Immunoperoxidase Secondary Detection System) as described elsewhere (29-36).

The mean percentages of positively stained cells were calculated for all the different lesions present in each sample. These values were tabulated for each group of animals (control group; experimental groups A, B, C) and every group’s percentages were compared with those corresponding to the previous group. In order to evaluate the pattern of antibody expression in relation to the histological status, the various lesions were divided according to tumor progression into a) normal tissue, b) non-cancerous and precancerous conditions (hyperplasia, dysplasia), c) tumor (early invasion, well-differentiated carcinoma, moderately differentiated carcinoma). In every lesion, the percentage of positively-stained cells from each non-cancerous and precancerous category was compared with those of the normal tissue, while the percentage of positively stained cells from each tumor category was compared with the average percentage of the non-cancerous and precancerous lesions.

Effect of Diabetes on Signal Transduction Pathways
Deregulation and mutations that augment the function of oncogenes, or the loss or inactivation of tumor suppressor genes, can directly enhance the potential of the cell to grow beyond its normal limits (37). These events can occur at several distinct points in the signal transduction cascades within cells. They include constitutive activation of receptor tyrosine kinase receptors (RTK), changes in the expression of second messengers and mutations in transcription factors which regulate cell proliferation and division, resulting in cancer progression (37).

The most common pathways leading to proliferation act through RTKs (38-40). RTKs bind to extracellular mitogenic growth factors and transduce these positive growth signals to intracellular second messengers (37). The substrates that RTKs phosphorylate belong to a class of proteins having Src homology 2 (SH2) domains (41). Some of these proteins, which play a critical role in cell signalling, are phospholipase-γ (PLCγ), phosphatidylinositol kinase (PI3K) and Grb2 (42, 43).

PLC-γ generates the second messengers diacylglycerol (DAG) and inositol triphosphate (IP3) by hydrolyzing membrane phospholipids (44). PI3K catalyzes the formation of phosphatidylinositol triphosphate (PIP3) resulting in the ultimate activation of protein kinase C (45, 46). Grb2 binds to the RTK and also interact with Son of Sevenless (SOS), which activates Ras (47-51).

Localization of Ras to the inner surface of the cytoplasmic membrane activates several downstream effectors, most notably the kinase Raf, which is the first signalling element in the mitogen activated protein kinase (MAPK) pathway (52). Upon activation, ras phosphorylates and thus activates the MAPKs (52). MAPKs in turn lead to the phosphorylation of other kinases eventually resulting in the enhanced expression of genes coding for proteins involved in the regulation of transcription whose products regulate cell proliferation and apoptosis (53).

In the rat model study of oral oncogenesis, the expression of several proteins implicated in these signal transduction pathways was detected by employing immunohistochemical methods. The studied protein factors included tyrosine kinase receptors (EGFR, erbB2, erbB3, FGFR-2, FGFR-3), cytoplasmic proteins (H-ras, N-ras), apoptosis-related proteins (Bax, Bcl-2), cell proliferation markers (Ki-67), nuclear transcriptional factors (p53, c-myc, c-fos, c-jun, ets-1), and cell cycle proteins (p16). The findings of these studies will be presented in regard to the involvement of signal transduction factors in every stage of oncogenesis. The signal transduction pathways that were affected in each sequential stage are presented in Figure 1.

Receptor tyrosine kinases. EGFR, HER-2/neu (erbB2) and HER-3 (erbB3) are included in the EGFR family of tyrosine kinase receptors. Overexpression of EGFR has been described in cases of head and neck cancer (54, 55). In addition, significantly high levels of EGFR have been closely correlated with tumor size and stage in head and neck SCC (56-59). Overexpression of erbB2 is significantly correlated with shorter survival of patients with oral SCC (60, 61). erbB3 Overexpression has been related to the malignant progression of head and neck SCC both in cell lines and clinical studies (61-64). Concerning FGFR-2 and/or FGFR-3 immunoexpression in oral cancer, a study suggested increased intensity of both receptors in dysplasias and squamous cell carcinomas (65).

In the rat model, a trend for reduced EGFR expression in diabetic compared to normal rats was revealed throughout oral oncogenesis (Figure 1) (31). The comparison of diabetic versus normal animals revealed that diabetes enhances the expression of erbB2 during nearly all stages of oral cancer development, as well as the expression of erbB3 in non-cancerous and precancerous stages of oral oncogenesis (Figure 1) (35). On the contrary, based on our findings, it seems that diabetes does not affect the FGFR-2 and FGFR-3 pattern of expression throughout the various stages of oral oncogenesis (Figure 1) (34).

Cytoplasmic proteins. Mutations of ras in oral cancer have been studied by a few other groups. It seems that these mutations are quite rare in Western populations and more
frequent in studies conducted with patients from Southeast Asian countries such as India and Taiwan (66). On the other hand, involvement of N-ras is an infrequent occurrence in the malignant progression of oral epithelial cells (67, 68). There are conflicting reports in the literature regarding the increase of N-ras expression at an early or a later stage in oral carcinogenesis (69, 70).

In our experimental model, a trend for increased H-ras expression was observed in diabetic compared to normal rats during oncogenesis; expression rose significantly in early invasion and well-differentiated OSCC (Figure 1) (31). N-ras expression increased with tumor advancement in diabetic rats, while in normal rats N-ras was not detected in the initial stages of oral oncogenesis and increased only in well-differentiated OSCC (Figure 1) (32). It seems that both H-ras and N-ras are positively affected by diabetes during oral oncogenesis.

**Apoptosis related-proteins and cell proliferation markers.** Bcl-2 and bax are two important effector genes during the apoptosis process. Certain studies of human OSCC biopsies reported increased levels of Bel-2 as well as increased levels of Bel-2/Bax ratio corresponding to reduced apoptosis in comparison to normal oral mucosa (71, 72). Other studies of human biopsies detected reduced Bel-2 expression, a reduced Bel-2/Bax ratio and increased apoptosis in the initial tumor stages, while Bax was not found to be significantly altered during the different stages of oral oncogenesis (73, 74).

Based on our findings in the rat model, the Bel-2/Bax ratio was relatively stable during the oncogenesis process in diabetic rats, contrary to normal rats which appear to have an increased Bel-2/Bax ratio in the stage of moderately differentiated carcinoma, indicating reduced apoptosis levels (Figure 1) (29). On the contrary, the expression of the cell proliferation marker Ki-67 was higher in diabetic rats than in normal ones in almost all stages of oral oncogenesis (Figure 1) (29). It is suggested that diabetes results in increased cell proliferation during oral oncogenesis, but this is accomplished without affecting the Bax/Bel-2-mediated apoptotic pathways.

**Nuclear transcriptional factors.** Transcription factors, or proteins that regulate the expression of other genes, are also altered in oral cancer. The transcription factor c-myc, which takes part in the regulation of cell proliferation and differentiation, is frequently overexpressed in oral cancers (75, 76). Overexpression due to gene amplification of c-myc is most frequently associated with poorly differentiated tumors and with poor prognosis (77). Gene mutations of p53 have been detected in more than 60% of cases of OSCC (78). The inactivated mutant p53 protein is more stable than the wild-type p53 protein, therefore its accumulation seems to occur early in oral neoplastic development and gradually increases through the sequential oncogenic stages from hyperplasia to OSCC (79, 80). Together c-fos and c-jun form the composite transcription factor activating protein-1 (AP-1), a mitogen-activated transactivator important for cell proliferation and differentiation (81). There are rare and conflicting reports in the literature regarding c-fos and c-jun expression in OSCC. In general, c-jun is considered as an early marker of differentiation and there are very few studies regarding its role in early carcinogenesis (82). Regarding c-fos expression, some studies have reported elevated levels of c-fos in malignant oral lesions (dysplasia, OSCC), others have noted maintained expression at normal levels during carcinogenesis, while still other studies have reported a high c-fos expression in normal oral mucosa followed by gradual decrease in the advanced stages of oral cancer (83-86). Up-regulation of ets-1 expression has been documented in many types of human tumors, including oral cancer. Generally, expression levels of ets-1 correlate well with the grade of invasiveness and metastasis (87).

The findings of the animal model suggested that diabetes contributed to increased accumulation of mutations in the p53 gene contributing to increased proliferation of tumor cells during oral oncogenesis (Figure 1) (30). Additionally, diabetes appeared to enhance c-myc expression only in the initial stages of oral oncogenesis (Figure 1) (30). Higher c-jun levels were observed in non-cancerous and precancerous stages in normal rats compared to diabetic rats, indicating that diabetes might not affect the c-jun N-terminal kinase (JNK)/c-jun pathway (Figure 1) (36). Interestingly, no statistical differences concerning c-fos expression were detected between diabetic and normal animals. Therefore, c-fos may not be implicated in the diabetes-related oncogenesis in the oral region of the present experimental animal system (Figure 1) (31). The same
pattern of elevated ets-1 expression was observed both in diabetic and normal rats, but in cancerous stages this expression was higher in diabetic than in normal rats indicating that diabetes may contribute to enhanced invasion and metastatic potential by increasing ets-1 levels (Figure 1) (32).

**Cell cycle regulators.** p16, a CDK inhibitor, binds to CDK4 and inhibits cyclin D-CDK4 activity. In addition, p16 is also negatively regulated by pRB (88). Although point mutations of the p16 gene in head and neck cancer are rare, alternative mechanisms of the abrogation of p16 function involving homozygous deletions or methylation of the 5-CpG promoter region of p16 have frequently been identified, suggesting that functional inactivation of p16 is a common event (89-91). In the experimental rat model of oral oncogenesis, the expression of the cell cycle regulator p16 was not found to be affected by diabetes (Figure 1) (33).

**Conclusion**

It is known that activation of insulin receptor as well as other types of cell receptors (EGFR, erbB2, erbB3, FGFR-2, FGFR-3) leads to the recruitment of signalling molecules, such as IRS-1, to the cell membrane, and the initiation of signalling cascades including the Ras/Raf/MAP kinase cascade and PI3 kinase cascade. A previous experimental study by our group suggested that the expression of IRS-1 was significantly higher in diabetic than in normal animals (28). Given the association between IRS-1 and H-ras, it follows that in diabetes, both H-ras and N-ras are elevated due to increased levels of IRS-1, leading to the activation of MAP kinase cascade. In our study it is obvious that both H-ras and N-ras play a very crucial role in the development of oral cancer in diabetic animals. Therefore, diabetes seems to promote the activation of Ras/Raf/MAPK signal transduction pathway by induction of receptors erbB2 and erbB3 mainly (and not EGFR) leading to increased cell proliferation. Additionally, in diabetic animals the Bcl-2/Bax ratio was found to be relatively stable during the oncogenesis process, indicating that diabetes has no effect on apoptosis levels.

It seems that animal models are a very useful tool towards understanding important mechanisms in oral oncogenesis. The use of carcinogens in animal models in order to study the mechanisms of oral oncogenesis is warranted, since chemical agents appear to be the dominant etiological factors in oral cavity.

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