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Oral Carcinogenesis Is not Achieved in Different Carcinogen-treated PAI-1 Transgenic and Wild-type Mouse Models

DIMITRIS AVGOUSTIDIS[1], THEMISTOKLIS NISYRIOS[1], EMEKA NKENKE[2], ROGER LIJNEN[3], VASSILIS RAGOS[1], DESPINA PERREA[4], ISMINI DONTA[4], APOSTOLIA VAENA[1], CHRISTOS YAPIJAKIS[1,5] and ELEFTHERIOS VAIRAKTARIS[1]

[1]*Department of Oral and Maxillofacial Surgery, University of Athens Medical School, Attikon Hospital, Athens, Greece;*
[2]*Department of Oral and Maxillofacial Surgery, University of Erlangen-Nuremberg, Erlangen, Germany;*
[3]*Center for Molecular and Vascular Biology, Catholic University of Leuven, Leuven, Belgium;*
[4]*N.S. Christeas Laboratory for Experimental Surgery and Surgical Research, University of Athens Medical School, Goudi, Athens, Greece;*
[5]*Department of Neurology, University of Athens Medical School, Eginition Hospital, Athens, Greece*

Abstract. *Aim: In an effort to assess the role of plasminogen activator inhibitor-1 (PAI-1) in oral squamous cancer development and progression, two different carcinogen treatment protocols were conducted. Materials and Methods: Protocol I included mice from a PAI-1 transgenic (Tg) breed (n=56) and their wild-type (WT) counterparts (n=56), divided into one control group and two main experimental groups, treated with 7,12-dimethylbenz[a]anthracene (DMBA) for 8 and 16 weeks, respectively. Protocol II included the same number and types of animals and groups, which were similarly treated with 4-Nitroquinoline 1-oxide (4-NQO) in drinking water. Two drugs that affect plasma PAI-1 levels, enalapril and pravastatin, were administered to certain subgroups of animals in both protocols. Results: None of the animals developed macroscopically-visible oral cancer lesions. Eleven animals under Protocol I and 52 animals under Protocol II died. Skin lesions were noted only in DMBA-treated animals (n=9). Almost all animals administered with 4-NQO developed alopecia and lost weight, while two of them developed stomach tumours, and one female mouse developed a large ovarian cyst. Conclusion: Transgenic mice may respond differently when used in well-established carcinogen models and oral carcinogenesis is hard to achieve in these rodents.*

Correspondence to: Professor Eleftherios Vairaktaris, M.D., D.D.S., Ph.D., Department of Oral and Maxillofacial Surgery, University of Athens Medical School, "ATTIKON" Hospital, Rimini 1, Chaidari GR – 12462, Athens, Greece. Tel: +30 2105831544, Fax: +30 2105831545, e-mail: lvairakt@med.uoa.gr

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The oral cavity constitutes one of the most common sites for cancer development. More specifically, oral cancer remains the sixth most common type of cancer and one of the leading causes of death, particularly in developing countries (1). Oral squamous cell carcinoma (OSCC) encompasses more than 90% of oral malignancies and has been reported in all parts of the oral cavity (2). Approximately 350,000 people are diagnosed with this disease annually worldwide, while the 5-year survival rates are poor, ranging between 30-50% in different studies and have not changed during the past two decades (3, 4).

The development and progression of carcinogenesis in the oral region is a multistep process in which mutations in oncogenes and tumour suppressor genes are involved, influenced by certain habits such as smoking, alcohol abuse and betel quid chewing (2, 3, 5). Furthermore, certain viruses such as human papilloma virus (HPV) are emerging as major risk co-factors, especially in patients of younger ages (2).

In an effort to understand the molecular mechanisms of oral cancer development and thus to improve the prognosis and treatment of the disease, several animal models for OSCC development have been generated (2). Although no animal model applies to any kind of human cancer perfectly, experimental animal models are of crucial importance to cancer research in general (2, 3, 6). Among different species of animals, oral cancer development has been achieved in hamsters, rats, mice and even zebrafish (2, 4, 6). Chemically-induced carcinogenesis has been applied in most models, while others include transplantable tumours, certain implant placement in soft tissues of the oral cavity, co-carcinogenicity models and the use of transgenic animals (2). Nevertheless, only a few models are well-established or have been confirmed by other studies.

Platelet activator inhibitor–1 (PAI-1), also known as serpine1, is emerging as a potential factor with an active role in oral carcinogenesis (7-10). Many authors have correlated plasma PAI-1 levels with the progression and development of oral cancer, although the mechanisms of its action are not yet fully understood (7-10). In an effort to further examine the role of PAI-1 in oral carcinogenesis, two different carcinogen-treated experimental animal models were used in the current study, each consisting of transgenic (Tg) mice hyperexpressing PAI-1 and their wild-type (WT) counterparts. The two strategies attempted to establish oral cancer lesions on these animals using: i) direct application of 7,12-dimethylbenz[a]anthracene (DMBA) in the oral cavity of the mice and ii) 4-Nitroquinoline 1-oxide (4-NQO) in drinking water. Both strategies were based on previous reports of successfully induced oral carcinogenesis in rodents (2, 3, 6, 11-13).

The present article discusses the results from the aforementioned different carcinogen-treated Tg and WT mouse models in comparison to previous studies including rodents for oral cancer development.

Materials and Methods

Animal characteristics. Three Tg PAI-1 mouse couples (*Mus musculus*, aP2 - PAI - Poly A 388, 50% FVB - 50% Bl6, Heverlee), as well as their respective WT counterparts, ranging from 8 to 16 weeks of age, were transferred to the N.S. Christeas Laboratory for Experimental Surgery and Surgical Research of the University of Athens Medical School, from the Laboratory Animal Centre of the Catholic University of Leuven. The animals were handled according to Greek legislation for the Care and Use of Laboratory Animals (P.D. 160/91 and Law 2015/92, conforming to European Directive 86/609) under controlled conditions with a 12-h light/dark cycle and maintained on a standard laboratory animal diet with constant nutrition (Mucedola s.r.l., Milan, Italy), supervised by an experienced veterinary surgeon. These animals were used for breeding.

Experimental protocol I. This protocol included animals recruited from the PAI-1 Tg and WT breeds and was carried out at two sequential time periods. In total, 112 male and female mice were randomly selected at the age of five weeks and divided into different experimental groups. The tongues of the animals were painted with 0.5% DMBA (Sigma-Aldrich Chemie GmbH, Germany), diluted in paraffin oil and stored at 4˚C. Disposable dental microapplicators were used for tongue painting, ensuring standard dosage of DMBA solution per animal. Both Tg and WT breeds were divided into one control group (no intervention, n=8) and the experimental groups A (n=24, 8 weeks' DMBA application) and B (n=24, 16 weeks' DMBA application). The animals from groups A and B were further categorised into four subgroups (A1, A2, A3, A4 and B1, B2, B3, B4) each including six mice. After completion of carcinogen treatment, animals from subgroups A1 and B1 were immediately euthanized, while animals from the other subgroups were sacrificed after 4 weeks of observation (subgroups A2 and B2), administration of enalapril (Renitec©, Vianex S.A., Greece) at a concentration of 12 mg/kg in drinking water for 4 weeks (subgroups A3 and B3), or administration of pravastatin (Pravachol©, Bristol – Myers Squibb Co., USA) at a concentration of 20 mg/kg in drinking water for 4 weeks (subgroups A4 and B4). Enalapril and pravastatin are known to affect plasma PAI-1 levels (14). The tongues of all animals were removed for pathological examination, as well as every lesion that was macroscopically visible in the head and neck region.

Experimental protocol II. Another generation of 112 male and female animals from the Tg and WT strains were used in this protocol, with the same characteristics as in protocol I and were divided into the same experimental groups. The carcinogen treatment for this protocol was carried out with 4-NQO (Sigma-Aldrich Chemie GmbH, Germany) diluted in the drinking water of mice at a standard concentration (100 μg/ml), based on the work of Tang *et al*. (3). All animals had free access to water supply and the solution of 4-NQO was renewed once a week. The control and experimental groups did not differ from those used in protocol I.

Results

Experimental protocol I. The results of this protocol are presented in Table I. During the phase of carcinogen treatment, 10 animals died unexpectedly, as well as one animal from the WT control group. All animals were clinically examined at different phases of this protocol. The clinical examination included observation of the cheek pouches, tongues and palate after anaesthetization, with the help of a magnifier lens. Eight animals from group B (5 Tg mice and 3 WT mice) developed small exophytic skin lesions in the genal and submental areas of the face, with the characteristics of papillomas. One Tg animal developed skin ulcer in the submental area. None of the animals presented with macroscopically visible tumours in the oral cavity (neither in cheek pouches, tongue, floor of mouth nor palate).

Experimental protocol II. The results of this protocol are presented in Table II. During the phase of carcinogen treatment, almost half of the animals died (52 mice), including all mice from group B. Gradually the majority of the 4-NQO-treated mice started developing hair loss (alopecia) from the frontal area of the neck, the thoracic and abdominal areas, and began losing weight. Their behavioural and movement patterns declined from normal; they became less active and developed movement disturbances and weakness over time. After the completion of the carcinogen treatment they regained movement and hair loss was drastically ameliorated. One WT female mouse from group A developed a large tumour in the abdominal area. After dissection and resection of the tumour, the pathological examination revealed a large ovarian cyst. Two animals developed solid tumours in the gastrointestinal system (stomach tumours). None of the animals presented with macroscopically visible tumours in the oral cavity (neither in cheek pouches, tongue, floor of mouth nor palate), nor with lesions on the skin of head and neck and the abdominal area.

Table I. *Experimental groups and outcome from 7,12-dimethylbenz[a]anthracene (DMBA) application to platelet activator inhibitor–1 (PAI-1) transgenic (Tg) and wild-type (WT) mice - Protocol I.*

	Control group (n=8) no DMBA application		Group A (n=24) 8-week DMBA application		Group B (n=24) 16-week DMBA application	
	Outcome	Intervention	Subgroups	Outcome	Subgroups	Outcome
PAI-1 Tg Mice (n=56)	No animals with visible lesions of oral cavity or skin	None	A1 (n=6)		B1 (n=6)	One animal died
		Observation (4 weeks)	A2 (n=6)	One animal died	B2 (n=6)	Two animals died Two with skin lesions One with skin ulcer
		Enalapril (12 mg/kg, 4 weeks)	A3 (n=6)		B3 (n=6)	One animal died Two with skin lesions
		Pravastatin (20 mg/kg, 4 weeks)	A4 (n=6)	One animal died	B4 (n=6)	One with skin lesions
WT Mice (n=56)	One animal died	None	A1 (n=6)		B1 (n=6)	One with skin lesions
		Observation (4 weeks)	A2 (n=6)		B2 (n=6)	Two animals died One with skin lesions
	No animals with visible lesions of oral cavity or skin	Enalapril (12 mg/kg, 4 weeks)	A3 (n=6)	One animal died	B3 (n=6)	One with skin lesions
		Pravastatin (20 mg/kg, 4 weeks)	A4 (n=6)		B4 (n=6)	One animal died

Table II. *Experimental groups and outcome from 4-Nitroquinoline 1-oxide (4-NQO) administration to platelet activator inhibitor–1 (PAI-1) transgenic (Tg) and wild-type (WT) mice - Protocol II.*

	Control group (n=8) no 4-NQO administration		Group A (n=24) 8-week 4-NQO administration		Group B (n=24) 16-week 4-NQO administration	
	Outcome	Intervention	Subgroups	Outcome	Subgroups	Outcome
PAI-1 Tg Mice (n=56)	No animals with visible lesions of oral cavity or skin	None	A1 (n=6)		B1 (n=6)	All animals died
		Observation (4 weeks)	A2 (n=6)	One animal died	B2 (n=6)	All animals died
		Enalapril (12 mg/kg, 4 weeks)	A3 (n=6)		B3 (n=6)	All animals died (One animal with stomach tumour)
		Pravastatin (20 mg/kg, 4 weeks)	A4 (n=6)	One animal died	B4 (n=6)	All animals died
WT Mice (n=56)	No animals with visible lesions of oral cavity or skin	None		A1 (n=6)	B1 (n=6)	All animals died
		Observation (4 weeks)	A2 (n=6)	One animal with large ovarian cyst	B2 (n=6)	All animals died
		Enalapril (12 mg/kg, 4 weeks)	A3 (n=6)	One animal died One with stomach tumour	B3 (n=6)	All animals died
		Pravastatin (20 mg/kg, 4 weeks)	A4 (n=6)	One animal died	B4 (n=6)	All animals died

Discussion

In an effort to shed light on the different pathways and susceptibility factors leading to oral cancer development and progression, animal models treated with carcinogens are widely used for conducting experimental protocols of oral oncogenesis. Two of these protocols are worldwide known for their efficacy in oral cancer development: a) the Syrian hamster cheek pouch model, firstly-introduced by Salley back in the 1950s, with DMBA application to the cheek pouches of the animals (15), and b) the rat oral cavity model introduced by Wallenius and Lekholm in 1973 with 4-NQO treatment of the palates of rats (16).

Morris standardized the first protocol in 1961 by showing that the pouch epithelium of younger hamsters was more susceptible to effects of DMBA and the five-week-old animals were the best candidates for commencing oral carcinogenesis experimental protocols with DMBA (17). As far as the second protocol is concerned, it was standardized in mice by Steidler and Reade and in rats by Wong and Wilson in 1983-84 (18, 19). Taking mice into consideration, the 4-NQO palate painting method results in a relatively low number of tumours and is quite laborious, therefore a new model for 4-NQO treatment with dilution in drinking water was introduced by Tang *et al*. in 2004 (3).

In an effort to procure OSCC in PAI-1 Tg mice and their WT counterparts in order to study the effects of PAI-1 on oral cancer, the cheek pouch protocol was applied first. To our knowledge, this protocol was imposed here for the first time in PAI-1 Tg. The carcinogen treatment was conducted for 8 and 16 weeks in different animal groups, so as to obtain tissues of different stages of oral oncogenesis. However, no macroscopically-visible lesions or exophytic tumours occurred in the oral cavity. The relatively low number of animals with lesions and exophytic tumours of the skin can possibly be explained by the fact that animals tend to lick different areas of their bodies with their tongues, such as the perioral or abdominal areas, thus spreading DMBA immediately after application to their tongues. Although resistant to developing skin cancer with DMBA treatment, skin carcinogenesis has been achieved in murines (20).

Since protocol I did not lead to oral cancer development, a new protocol based on 4-NQO in drinking water was applied to a new generation of Tg and WT mice. This protocol was based on the work of Tang *et al*. (3), with a 4-NQO concentration of 100 μg/ml in drinking water. This concentration was selected since the authors indicated that 100% of the mice developed lesions in the oral cavity (dysplastic or hyperplastic areas, exophytic mostly papillary tumours, papillomas and invasive OSCCs) after a period of either 8 weeks or 16 weeks of treatment, while four out of five mice survived at week 24 after the initiation of the experiment (3). Moreover, the animals in our study had free access to drinking water, as in the work of Tang *et al*. (3).

However, in our protocol no macroscopically-visible lesions were observed either in the oral cavity or the skin of the animals. At the same time, a large number of mice treated with 4-NQO died (46% of the total animals) and almost all developed alopecia and weight loss, as well as behavioural and movement disturbances in terms of weakness, as previously stated. Moreover, one female mouse developed a large ovarian cyst. Alopecia and weight loss have also been reported in 4-NQO-treated mice and were attributed to the clinical status of dysphagia due to the induction of masticatory system impairment by oral cancer, leading to a low protein intake (21). However, this does not seem a plausible explanation in this case, since weight loss and dysphagia occurred within a month of the 4-NQO administration, while no oral lesions were apparent, and weight was gradually regained after the carcinogen administration was ceased. Moreover, 4-NQO is a well-known mutagen, clastogen and carcinogen, and may cause toxicity in multiple organs, including the stomach, liver and ovaries, as well as in blood cells (22, 23). This would explain all clinical signs and symptoms that the murines developed during 4-NQO administration.

In conclusion, it must be stated that transgenic mice may respond differently in well-established carcinogen models and oral cancer development is still hard to achieve. Moreover, as far as oral cancer protocols with carcinogen treatment in rodents are concerned, the hamster cheek pouch model with DMBA application and the rat tongue model with 4-NQO treatment still remain the golden standard for most experimental studies (2, 24).

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