Apoptin gene therapy in head and neck cancer

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Induced oral epithelial dysplasia in the mouse model

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Submitted

Abstract

Oral dysplasia is a frequent pre-malignant lesion. Preventing these pre-malignant lesions to develop into malignancy is the main goal of treatment. Optimal treatment is debated, and a reliable animal model could be helpful to elucidate pathogenesis and to test treatment modalities. The carcinogen 4-nitroquinoline 1-oxide (4NQO) induces clinically representative squamous cell carcinoma of the oral mucosa in mouse; however, less is known whether the carcinogen also leads to pre-malignant lesions. To this end we studied the microscopic changes in the mucosa of the mouse tongue after treatment with 4NQO. 4NQO was applied orally in male CBA mouse three times a week during 16 weeks. Architectural and cytological features, used in human pathology are used to describe the degree of dysplastic changes. The successive stages of epithelial dysplasia, mild, moderate and severe dysplasia were seen. All three stages contained architectural and cytological features seen in human dysplasia, which implies that the carcinogenic mouse model resembles that of the human counterpart, making it apt for laboratory investigation.

Introduction

Squamous cell carcinoma of the tongue is the most common oral cavity malignancy. The incidence of tongue tumor has risen with 16% in the period between 1975 and 2001[1]. The occurrence of these oral malignancies is strongly connected with smoking tobacco and consumption of alcohol[2]. The treatment of locoregional tumors of the oral cavity often has a profound effect on the quality of life. Besides cancer, clinicians are confronted with pre-malignant lesions; those require a totally different approach and treatment.

Epithelial dysplasia mostly appears as a white, red or as a mixed red and white lesion. There is an association between the risk of malignant transformation and the histology of the lesion[3]. Well documented studies with a mean follow up of more than seven years revealed that the overall malignant transformation rates for dysplastic lesions range from 9 to 36%[4]. Interestingly the malignant transformation rate for mild epithelial dysplasia is similar to that of severe dysplasia[5]. The primary goal of management of pre-malignant lesions is preventing these lesions to transform in to malignant tumors. There is no consensus on what the best treatment is of oral dysplasia in order to prevent the development of malignancies. The most recent Cochrane review concluded that there is a complete lack of qualitative research for the treatment and management of dysplastic lesions including randomized controls trials nor is there consensus on the minimal required margins of surgical resection[6]. There is no evidence that non-surgical treatment such as topical Bleomycin[7] or Lycopene[8] is effective in preventing malignant transformation.

These uncertainties request for more (pre)clinical research. In animal models, epithelial dysplasia has been described, although the emphasis of these investigations lies more on squamous cell carcinoma[9-11]. Different animal models are known to develop oral dysplasia, including hamster[9], rats[12], and mice[11] models. The hamster cheek pouch model, in which 7,12-dimethlbenz(a)anthrance is used to induce tumors, is unattractive

because the tumors are not the same as their human equivalents[13]. The oral induced dysplasia and squamous cell carcinoma by 4-nitroquinoline-1-oxide (4NQO) in rats has been described[12,14] and it revealed that there is close similarity between induced dysplasia of the palate mucosa and that of human oral epithelial dysplasia. For investigating dysplasia of the tongue by 4NQO application, mice seem more appropriate than rats because they consistently develop more advanced premalignant changes and earlier squamous cell carcinomas than the palate[15], whereas in rats the palate is most of the times the first affected subsite. In contrast to the rat palate model[14-17], for the 4NQO mouse model it is unknown whether the expressed dysplasia on the tongue is the same, as the human equivalent and thus apt for in-vivo investigation. The time that is needed for dysplastic lesions to develop is accurately known, but the histopathology up till now is not thoroughly been described.

The aims of this study are:

- to describe the effectiveness of the 4NQO model
- to study the histological characteristics of carcinogenic induced dysplasia of tongue of the mouse
- to compare the lesions to dysplastic lesions of human oral epithelium
- to explore the potential of the 4NQO model to study new therapies of premalignant lesions and squamous cell carcinoma of the oral mucosa.

Materials and Methods

Animals and carcinogen.

CBA male mouse, 7-8 weeks of age and weighing between 23 and 27 grams (Harlan, Zeist, The Netherlands), were kept at standard laboratory conditions of alternating 12-h periods of light and darkness and were fed sterilized laboratory chow and water ad libitum. All experiments were approved in advance by the Leiden University Animal Welfare Committee. The carcinogen 4NQO was dissolved in propylene glycol to a final concentration of

5 mg/ml. A fresh aliquot was used for each application session and anesthesia was achieved by inhalation of halothane vapor. The tongue was stroked once with a brush, which had been dipped once in the 4NQO solution. The mice were restrained from drinking the first hour after 4NQO application. All mice were carefully inspected daily and weighed weekly. The mouse was brushed with 4NQO during 16 weeks with a frequency of 3 weekly treatments. Mouse showing signs of morbidity were immediately sacrificed.

Histopathology and Immunohistochemistry.

Prior information showed that dysplasia was achieved between 4 and 12 weeks after the final 4NQO application and squamous cell carcinoma after 20 weeks. Twelve mice were sacrificed between 4 and 12 weeks and 4 after 24 weeks. At these time points tissue was removed and fixed in freshly made 4% buffered formalin. After paraffin embedding, serial sections of 4 μm width were made and sections were stained with hematoxylin-eosin (HE). The epithelium was examined by using architectural and cytological features used to diagnose human dysplasia (Table 1). This index involves the evaluation of 7 architectural and 9 cytological characteristics

Results

Twelve mice were sacrificed between 20 and 28 weeks after starting the experiment. One of these had a normal epithelium of the tongue (Figure 1). The surface of the tongue is covered with keratinized stratified squamous cell epithelium containing numerous filiform papillae. The basal cell layer contains specialized cells for mitotic division and the parabasal cells are differentiating cells with a layer of highly keratinized cells. Mild dysplasia was diagnosed in 5 mice, moderate and severe dysplasia both in 3 animals. The scores of the features of dysplasia, both architectural and cytological of the 3 types of dysplasia are shown in Table 1.

Figure 1

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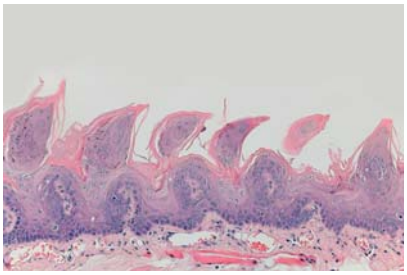

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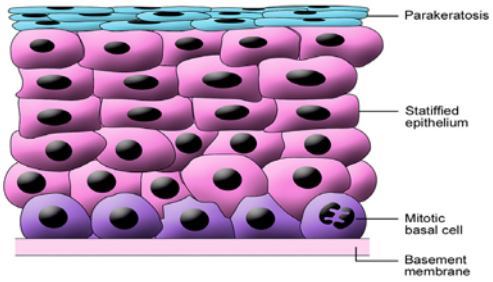

A. Microscopy of normal epithelia of the tongue of the mouse. (H&E, Original magnification x 100). B. Illustration of normal epithelia with an intact basal membrane, stratified epithelium and parakeratosis.

In mild dysplasia (Figure 2), the dysplastic changes are limited to the lower one-third of the epithelium. Basal cells show a lack of polarity, cells have slightly bigger hyperchromatic nuclea and there is an increased nuclear/cytoplasmatic ratio. Parakeratosis is covering the epithelia and there are abnormal mitotic figures. The upper cell layers show normal maturation.

Figure 2

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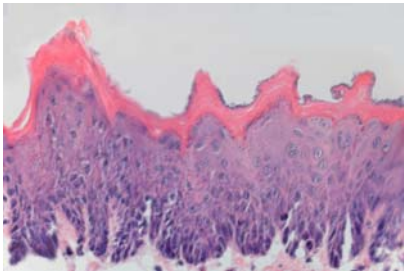

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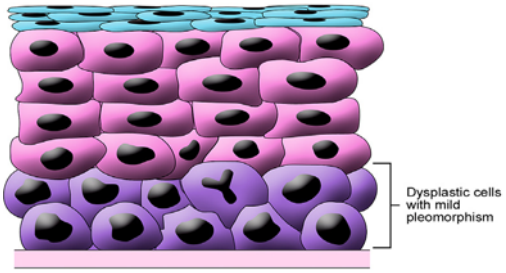

A. Histopathology 8 weeks after the final 4NQO application showing mild dysplasia. There is a disturbance of polarity in basal cells with hyperchromatic nuclea and an increased nuclear/cytoplasmatic ratio. Cells are anisocytosic and anisokaric. Atypical mitotic figures are increased and atypical. (H&E, original magnification x 100). B. Illustration of mild dysplasia with atypical changes in the lower one-third of the epithelia.

In moderate dysplasia (Figure 3), the alterations are limited to the lower two-thirds of the epithelium. The basal part of the epithelia shows an increase of loss of polarity, and irregular maturation. Enlarged pleomorphismic cells with hyperchromatic nucleus and enlarged nucleoli are visible. There is an obvious raise of mitotic figures, predominantly in the parabasal cell layers and an increase of nuclear-cytoplasmic ratio.

Figure 3

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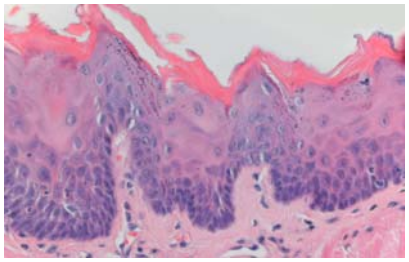

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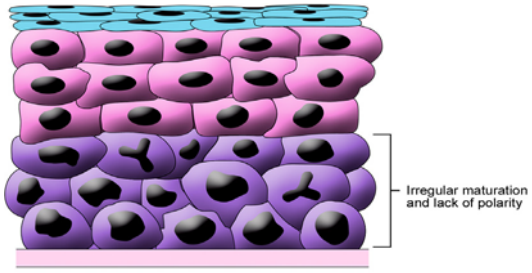

A. Moderate dysplasia of the tongue 10 weeks after final 4NQO application. Enlarged pleomorphismic cells with hyperchromatic nucleus stand out with irregular stratification and drop-shaped rete ridges. (H&E, original magnification x 100). B. Illustration of moderate dysplasia with irregular maturation and lack of polarity. Changes extend beyond the lower one-third of the epithelia.

In severe dysplasia (Figure 4) the atypical changes involve the full thickness of the epithelium in which there is considerable hyperplasia and a strong irregular maturation with strong cell atypia.

Figure 4

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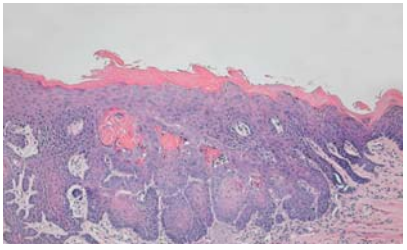

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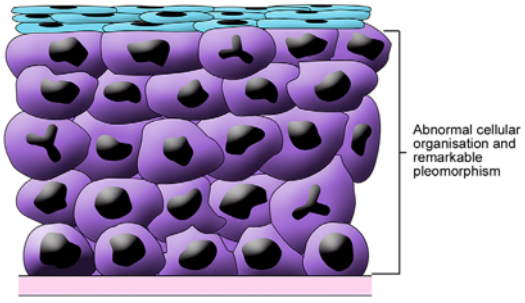

A. Histopathology showing severe dysplasia of the tongue 28 weeks after starting of the experiment. There is notable irregular stratification of the epithelia, loss of intercellular adherence and abnormal keratinization at the surface. (H&E, original magnification x 100). B. Illustration of moderate dysplasia with abnormal cellular organization and pleomorphism. Changes involve the full thickness of the epithelia.

Figure 5

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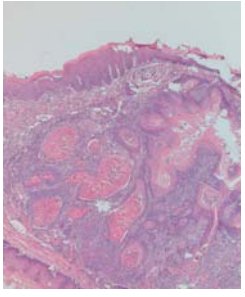

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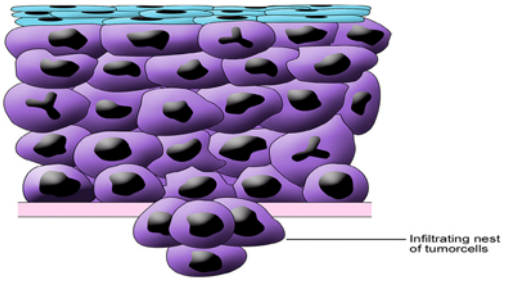

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There are an intensified number of mitotic figures and nuclear-cytoplasmic ratio. Anisocytosis and anisokary is widely present. Compared with mild and moderate dysplasia the lack of polarity is stronger. Cells have nuclear pleomorphism and hyperchromatism, with prominent larger nucleoli and there is an increased cellularity. Atypical mitotic figures are seen in large quantities. In the sample shown in figure 4 the full thickness of the squamous epithelia was involved, so in fact this represents carcinoma in situ.

At the end of the experiment 4 mice were sacrificed after 40 weeks and appeared to have an invasive squamous cell carcinoma (Figure 5).

Table 1 Microscopic findings of dysplasia

		Mild dysplasia	Moderate dysplasia	Severe dysplasia
Architectural features	Irregular epithelial stratification	-	++	++
	Abnormally superficial mitoses	-	+	++
	Loss of polarity of basal cells	+	++	+++
	Pre-mature keratinization in single cells	-	+	++
	Drop-shaped rete ridges	-	+	++
	Keratin pearls within rete ridges	-	-	++
	Increased number of mitotic figures	+	++	+++
Cytological features	Abnormal variation in nuclear size	+	+	+++
	Abnormal variation in nuclear shape	-	+	+++
	Abnormal variation in cell size	+	++	+++
	Abnormal variation in cell shape	+	++	+++
	Increased nuclear-cytoplasmic ratio	+	++	+++
	Increased number and size of nucleoli	+	+	+++
	Increased nuclear size	+	++	+++
	Atypical mitotic figures	+	++	+++
	Hyperchromasia	+	++	+++

Discussion

The present study was designed to evaluate the development of epithelial dysplasia and squamous cell carcinoma of the tongue in mouse after repetitive application of 4NQO. Both in rats and mice it has been proved that 4NQO induces dysplasia and squamous cell carcinomas. The emphasis has been predominantly on squamous cell carcinomas and only in rats there has been histopathological description of induced dysplasia. For the first time we minutely describe the several stages of oral dysplasia in mouse by using the architectural and cytological features, which is a useful and consistent method of the interpretation and quantification of epithelial dysplasia in human pathology. This classification makes use of 5 architectural and 4 cytological criteria for epithelial dysplasia, although it is unknown how many features are necessary for a particular diagnosis. Some of these features are present in squamous cell carcinoma. There is a continuous spectrum from mild, via moderate to severe dysplasia in which there is a quantitative increase in architectural and cytological characteristics wherein mild dysplasia is restricted to the lower one-third and severe dysplasia involves the full thickness. This full continuum was seen in our specimens. In mild dysplasia, except for increase of cellularity, all early changes were observed. As in the human equivalent the changes were limited to the lower third of the epithelia. In mild dysplasia the nuclear abnormalities stand out[18] and changes are limited to the parabasal layers[19]. In the moderate dysplasia the nuclear abnormalities are more marked and nucleoli are more prominent, changes that both were observed. In moderate dysplasia increased cellularity emerges and the changes expand to the middle third of the layers. Our observations of evident nuclear abnormalities, loss of maturation and mitosis present high up in the epithelia all are typical of severe dysplasia[20,21]. All nine features are abundantly present in severe dysplasia. The involvement of all layers in 3 specimens with severe dysplasia and an intact basement membrane justifies the diagnosis carcinoma in situ. The diagnosis carcinoma-in-situ

and the distinction between severe dysplasia is often difficult and sometimes arbitrary. The last 4 mice sacrificed at the end of this experiment were diagnosed with an invasive squamous cell carcinoma. Prior observations that eventually all mouse after the time point of 40 weeks bear squamous cell carcinoma and the fact that in this experiment the mouse sacrificed at the end of the experiment also were diagnosed with squamous cell carcinoma strengthen our believe that all dysplastic lesions in this animal model in time eventually develop in squamous cell carcinomas.

The histological characteristic of the oral epithelial dysplasia in mouse resembles that of oral dysplasia in humans. The gradual increase of the features in mild, moderate and severe dysplasia showed strong parallels to its human counterpart. Thus the 4NQO mouse model offers a suitable model to study oral premalignant and squamous cell carcinoma of the mouth. Current and new treatment modalities can very well be studied in this model.

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