Archives of Oral Biology 96 (2018) 169–177

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/archoralbio

Immunohistochemistry profile of p75 neurotrophin receptor in oral epithelial dysplasia and oral squamous cell carcinoma induced by 4-nitroquinoline 1-oxide in rats

Marina Gabriela Teixeira Buck[a], Priscila Souza Cabral Tuci[a], Flávia Cristina Perillo Rosin[a], Ana Rita Pinheiro Barcessat[b], Luciana Corrêa[a,*]

[a] Pathology Department, School of Dentistry, University of São Paulo, Av. Prof Lineu Prestes, 2227 – Cidade Universitária, 05508-000 São Paulo, SP, Brazil
[b] Biological Health Sciences Department, School of Nursing, Federal University of Amapá, Rod. Juscelino Kubitschek, KM-02 Jardim Marco Zero Macapá, 68.903-419 Macapá, AP, Brazil

ARTICLE INFO

Keywords:
p75 neurotrophin receptor
4-Nitroquinoline 1-oxide
Oral carcinogenesis

ABSTRACT

Objective: The 4-nitroquinoline 1-oxide (4-NQO) model for carcinogenesis has been used to investigate cancer stem cells (CSC), but no study has addressed the role of the p75 neurotrophin receptor ($p75^{NTR}$) in 4-NQO-induced oral dysplasia and oral squamous cell carcinoma (OSCC). The aim of this study was to evaluate the immunohistochemistry profile of the $p75^{NTR}$ during 4-NQO-induced oral carcinogenesis in rats and to verify whether this profile has an association with proliferating cell nuclear antigen (PCNA) immunolabeling.
Design: For 28 weeks, rats were exposed to 4-NQO, which was diluted in the drinking water. After 3, 5, 7, 16, and 28 weeks, the animals were euthanized and their tongues were histologically analyzed using $p75^{NTR}$ and PCNA immunolabeling.
Results: In animals without 4-NQO exposure, the $p75^{NTR}$ and PCNA were expressed only in the basal epithelial layer and in a clustered manner. The oral epithelium showed dysplasia and a significant increase in the number of $p75^{NTR}$- and PCNA-positive cells, which were localized mainly in the basal and suprabasal epithelial layers during weeks 5–16 of 4-NQO exposure. When the epithelium invaded the lamina propria and well-differentiated OSCC began, the $p75^{NTR}$-positive cell frequency drastically decreased in epithelial cords and nests, showing a negative correlation with PCNA expression. $p75^{NTR}$ immunolabeling during 4-NQO-induced carcinogenesis was similar to that described for human head and neck dysplasia and neoplasia.
Conclusions: $p75^{NTR}$ immunolabeling observed in 4-NQO-induced oral dysplastic and OSCC lesions were related to the early phases of oral carcinogenesis and may help predict cell dysplasia and malignant transformation.

1. Introduction

Oral squamous cell carcinoma (OSCC) has high frequency worldwide, exhibiting a multi-step process that includes a phase of oral epithelial dysplasia with different degrees of severity, followed by complete malignant transformation and evolution to OSCC (Choi & Myers, 2008). Experimental rat, mouse, or hamster models were developed to investigate the process of oral malignant transformation in a chronologic sequence. One of these models uses 4-nitroquinoline 1-oxide (4NQO), a chemical carcinogen that is a potent oxidant, inducing high production of reactive oxygen species that react with guanine and causes DNA strand breaks. The 4-NQO-induced DNA mutations lead to modifications during cell differentiation and proliferation, which culminates with induction of hyperplasia, dysplasia, and malignant neoplasia of the oral epithelium (Kanojia & Vaidya, 2006). One of the main advantages of the 4-NQO model is that the oral mucosa transformation can be monitored in accordance with the exposure time to the carcinogen, and each step of oral carcinogenesis can be investigated separately. Another advantage is that the histological pattern of 4-NQO-induced epithelial dysplasia is very similar to that observed in humans, allowing extrapolations to human conditions in the oral cavity (Nauta, Roodenburg, Nikkels, Witjes, & Vermey, 1995).

Applications of the 4-NQO model include investigation of putative OSCC prognosis biomarkers, such as proteins related to apoptosis, cell cycle, cell-cell interactions (Kanojia & Vaidya, 2006), and the efficacy evaluation of antineoplastic therapy, mainly chemotherapeutic agents.

* Corresponding author.
E-mail address: lcorrea@usp.br (L. Corrêa).

https://doi.org/10.1016/j.archoralbio.2018.09.010
Received 14 May 2018; Received in revised form 31 August 2018; Accepted 18 September 2018

Recently, studies have also investigated the expression of stem cell markers during 4-NQO-induced oral carcinogenesis, focusing on the role of each marker in accordance of the phase of malignant transformation (Lim et al., 2014; Tanaka et al., 2016), and on the effect of antineoplastic formulations in stem cells (Siddappa et al., 2017).

Cancer stem cells (CSCs) were shown to participate in OSCC pathogenesis and progression (Papagerakis et al., 2014), contributing with the tumor growth because of their potential for self-renewal and hierarchical differentiation (Prince et al., 2007). Many CSC biomarkers have been associated with OSCC progression, invasion, and metastasis, such as Sox2 (Li et al., 2014), ALDH1 (Zhou & Sun, 2014), and CD44 (Chen et al., 2014). These markers and others (Oct4, CD133, and Bmi1) were also addressed in oral dysplasia and OSCC induced by 4-NQO (Lim et al., 2014; Siddappa et al., 2017; Tanaka et al., 2016). No study has investigated the role of p75 neurotrophin receptor ($p75^{NTR}$) during oral carcinogenesis induced by 4-NQO.

$P75^{NTR}$ is a surface glycoprotein and a member of the tumor necrosis factor-receptor superfamily, which is involved in cell proliferation and survival in neural and non-neural tissues. $P75^{NTR}$ is a receptor for various neurotrophins, stimulating several signal transduction pathways related to apoptosis, cell survival, cell cycle regulation, cell migration and invasion, and progenitor differentiation (Tomellini, Lagadec, Polakowska, & Le Bourhis, 2014). Some studies have reported a stem cell phenotype of $p75^{NTR}$-positive cells in various tumors, such as melanoma (Civenni et al., 2011) and breast cancer (Tomellini et al., 2015). In the head and neck region, $p75^{NTR}$ has been described as an important marker for CSC in esophageal carcinoma (Huang et al., 2009; Yamaguchi et al., 2016), and OSCC (Kiyosue et al., 2013; Tong, Sun, Huang, Li, & Zhang, 2017). $p75^{NTR}$ expression was also associated with poor prognosis in OSCC, mainly with poor disease-free survival (Søland, Brusevold, Koppang, Schenck, & Bryne, 2008).

We conducted a literature survey on immunohistochemical expression of $p75^{NTR}$ in squamous cell carcinoma localized in the head and neck region, excluding glandular tumors (such as salivary gland tumors, thyroid tumors) and cutaneous neoplasms. In Table 1, we describe the previously reported immunolabeling pattern of $p75^{NTR}$ in normal, dysplastic, and neoplastic tissues in the head and neck region. No study has analyzed animal tissue; all the investigations involved biopsies or xenotransplantation of human tissues. In normal mucosa in the mouth, esophagus, hypopharynx, and larynx, $p75^{NTR}$ was expressed only in the basal epithelial layer (Abdulmajeed, Dalley, & Farah, 2013; Kiyosue et al., 2013; Mochizuki et al., 2016; Nakamura, Endo, & Kinoshita, 2007; Okumura et al., 2006). In dysplastic human lesions, $p75^{NTR}$ expression was variable based on the anatomic site. Some studies with head and neck tumors found $p75^{NTR}$ expression in cells localized in basal and suprabasal layers, with more intensity in patients with severe dysplasia (Abdulmajeed et al., 2013; Kiyosue et al., 2013; Mochizuki et al., 2016). For neoplastic lesions, the studies emphasized that poorly differentiated tumors had more intense $p75^{NTR}$ expression than well-differentiated tumors. Areas with keratin pearls were negative, and only peripheral cells in islets and nests localized in the infiltrative margin or in the invasion front were positive for this protein (Kiyosue et al., 2013; Kojima et al., 2017; Søland et al., 2008). Some of these studies associated the $p75^{NTR}$ expression with Ki67 immunolabeling (Osman et al., 2015), mainly in poorly differentiated squamous cell tumors (Mochizuki et al., 2016).

In the present study, we aimed to evaluate the immunohistochemistry profile of $p75^{NTR}$ in oral dysplasia and OSCC induced by 4-NQO in rats, and to verify whether there is an association between $p75^{NTR}$ expression and proliferating cell nuclear antigen (PCNA) immunolabeling. Our hypothesis was that $p75^{NTR}$ expression will increase in accordance with the transition from oral dysplasia to OSCC and that there will be a positive correlation between this marker and PCNA. This trend will be similar to that described previously for human squamous cell carcinoma in the head and neck region.

2. Materials and methods

The following methodology was approved by the Ethical Committee on Animal Research at our institution (Proc. N. 007/2013) and followed the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

2.1. Experimental groups

Male Wistar rats, 200 g mean body weight, were randomly divided into: a) Control group (n = 5), including animals without any treatment; b) Group 1 (n = 14), including animals treated with 4-NQO for 16 weeks; and c) Group 2 (n = 8), including animals treated with 4-NQO for 28 weeks.

The animals in all groups were maintained in individual cages in a laboratory with controlled temperature (24 ± 2 °C) and light-dark periods of 12 h. A commercial diet (Labina®, Purina, Brazil) and drinking water were offered *ad libitum*.

2.2. Induction of oral lesions with 4-NQO

4-NQO (Sigma Chemical Co., St. Louis, MO, USA) was diluted in drinking water (20 ppm), and 400 mL of this solution was administered to the animals for 16 weeks (Group 1) and for 24 weeks (Group 2). After this period, the animals of Group 2 received an additional dose of 5 ppm 4-NQO solution for 4 weeks. We adapted this protocol from a previous study (Minicucci et al., 2011). Generally, moderate to severe epithelial cell dysplasia can be observed after 12 weeks of treatment, and OSCC after 20 weeks of 4-NQO exposure. The animals in the Control group were maintained under the same conditions as for Groups 1 and 2, but the drinking water was free of 4-NQO.

2.3. Euthanasia and histological processing

The animals of Group 1 were euthanized after 3, 5, and 7 weeks (three animals in each period) and after 16 weeks (five animals) of 4-NQO exposure. The animals in Group 2 were euthanized after 28 weeks, by lethal dose of ketamine (Dopalen®, Vetbrands, Brazil) and xylazine (Anasedan®, Vetbrands, Brazil). Animals in the Control group were euthanized after 28 weeks. The tongue of all animals were then excised and fixed in 10% neutral-buffered formalin solution for 24 h. After fixation, the specimens were sectioned in the middle and routine histological processing and paraffin embedding were performed. Histological sections (5 µm thick) were stained with hematoxylin and eosin and analyzed by one pathologist, who described the characteristics of the tongue mucosa, mainly regarding epithelial atypia and presence of OSCC in the specimens.

2.4. Immunohistochemical analysis

Histological sections (3 µm thick) obtained from the specimens of all groups were stretched on glass slides treated with 3-aminopropyltriethoxysilane and maintained at 60 °C for 24 h. Dewaxing and dehydration were then performed with xylene and descending concentrations of ethanol solutions, respectively. Antigen retrieval was performed using 0.01 M citric acid solution, pH 6.0, at 95 °C for $p75^{NTR}$ and PCNA (30 min and 45 min, respectively). Endogenous peroxide was blocked using methanol and 3% hydrogen peroxide solution for 15 min, followed by the blockage of non-specific antigen sites with 1% bovine serum. Samples were incubated with the primary antibody against $p75^{NTR}$ (1:150, D4B3 clone, #8238, Cell Signaling, Danvers, Massachusetts, USA) for 1 h at room temperature. Samples were also incubated with the primary antibody against PCNA (1:50, PC10 clone, M0879, Dako Corporation, Carpinteria, CA, EUA) overnight at 4 °C. For $p75^{NTR}$, sections were incubated for 30 min with an immunoenzymatic polymer containing a secondary antibody (Histofine ® Simple Stain