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Nicotine induces oral dysplastic keratinocyte migration *via* fatty acid synthase-dependent epidermal growth factor receptor activation

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ABSTRACT

Despite advances in diagnostic and therapeutic management, oral squamous cell carcinoma (OSCC) patient survival rates have remained relatively unchanged. Thus, identifying early triggers of malignant progression is critical to prevent OSCC development. Traditionally, OSCC initiation is elicited by the frequent and direct exposure to multiple tobacco-derived carcinogens, and not by the nicotine contained in tobacco products. However, other nicotine-containing products, especially the increasingly popular electronic cigarettes (e-cigs), have unknown effects on the progression of undiagnosed tobacco-induced oral premalignant lesions, specifically in regard to the effects of nicotine. Overexpression of fatty acid synthase (FASN), a key hepatic *de novo* lipogenic enzyme, is linked to poor OSCC patient survival. Nicotine upregulates hepatic FASN, but whether this response occurs in oral dysplastic keratinocytes is unknown. We hypothesized that in oral dysplastic keratinocytes, nicotine triggers a migratory phenotype through FASN-dependent epidermal growth factor receptor (EGFR) activation, a common pro-oncogenic event supporting oral carcinogenesis. We report that in oral dysplastic cells, nicotine markedly upregulates FASN leading to FASN-dependent EGFR activation and increased cell migration. These results raise potential concerns about e-cig safety, especially when used by former tobacco smokers with occult oral premalignant lesions where nicotine could trigger oncogenic signals commonly associated with malignant progression.

1. Introduction

Approximately 410,000 new cases of oral cancer, largely oral squamous cell carcinoma (OSCC), are diagnosed each year worldwide with an estimated 146,000 deaths [1]. In the United States, 51,540 newly diagnosed OSCC tumors and 10,030 deaths are expected to occur in 2018 [2]. Although treatments have unquestionably improved in recent years, the 5-year OSCC survival rate remains at around 50% [1]. Heavy tobacco use continues to be the major risk factor for OSCC development, and the poor survival can be attributed to late diagnosis along with high recurrence rates following treatment [3].

Typically, the direct and frequent exposure of healthy oral mucosa to a myriad of tobacco carcinogens, present in combustible as well as smokeless tobacco products, may lead to the formation of potentially malignant lesions that histologically exhibit different degrees of epithelial dysplastic changes [3]. In fact, it is difficult to predict the transformation rate of oral epithelial dysplasias to OSCC, as it has been reported to be between 0.13% and 17.5% [4]. Thus, defining determinants of neoplastic progression is critical to the field of oral carcinogenesis.

The use of electronic nicotine delivery systems (ENDS), including a variety of vaping devices such as electronic cigarettes (e-cigs), is increasing at an alarming rate around the world [5]. Although nicotine is the main trigger for addiction to tobacco and tobacco-derived products, it is not considered a carcinogenic chemical *per se* [6]. Surprisingly, the effects of nicotine in the oral cavity have been largely understudied. Indeed, approximately 50% of current and former cigarette smokers, including individuals who are possibly developing potentially malignant oral dysplastic lesions, have started vaping nicotine-containing e-cigs [5]. To this end, there is a critical need to determine whether nicotine induces any pathobiological effect on oral dysplastic keratinocytes, especially if former heavy users of combustible and/or smokeless tobacco start using a vaporized form of nicotine as part of a smoking cessation intervention.

It has been recently reported that in OSCC nicotine affects tumor cell gene expression, apoptosis evasion, migration, proliferation and chemoresistance; yet there is little evidence available on the role of nicotine in oral precancer [7–12]. One study indicates that nicotine

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increases markers for early cancer progression in human oral keratinocytes, while another shows nicotine is capable of suppressing apoptosis in oral premalignant cells [13,14]. Considering the potentially large population of individuals with occult, undiagnosed oral premalignant lesions who are using nicotine-containing e-cigs, and the gap that exists on the basic understanding of the effects of nicotine on oral dysplastic keratinocytes, we designed this study to gain mechanistic insight into the role of nicotine in oral precancerous cells.

Interestingly, nicotine increases hepatic fatty acid synthase (FASN) expression and activity [15]. FASN is responsible for the final step of *de novo* lipogenesis, and is typically not expressed in non-hepatic tissues [16]. However, in the particular case of the oral mucosa, FASN is overexpressed in oral hyperkeratosis, dysplasia, and OSCC, and is associated with histological grade and risk of recurrence [16,17]. Moreover, FASN pharmacological inhibition reduces proliferation and migration by 50% in an *in vivo* OSCC model, implicating a role for FASN in OSCC cell behavior [18]. Furthermore, FASN appears to be very relevant to cancer since it has been linked to Epidermal Growth Factor Receptor (EGFR) activation [19–21]. In OSCC, EGFR overexpression and its aberrant pro-oncogenic signaling are strongly associated with tumor progression, advanced clinical stages and worse survival rate outcomes [3,22]. In fact, nicotine exposure causes EGFR-dependent growth and migratory signals in cancer cells [23–26]. Due to the significant pro-oncogenic relationship that FASN and EGFR signaling might play in oral carcinogenesis as it relates to the effects of nicotine both in hepatic and non-hepatic tissues, we hypothesized that oral dysplastic keratinocytes exposed to nicotine may acquire a migratory phenotype through FASN-dependent EGFR activation. Our data show that exposure of oral dysplastic keratinocytes to nicotine led to a marked increase in FASN protein expression and FASN-dependent EGFR phosphorylation and pathway activation. Furthermore, nicotine-stimulated FASN significantly increased oral dysplastic keratinocyte migration, a common hallmark of malignant cells. Collectively, this study provides novel insight into the role of nicotine on FASN/EGFR signaling and cell migration in oral precancerous cells.

2. Materials and methods

2.1. Cell culture and reagents

Human-derived dysplastic oral keratinocytes (DOK; Millipore Sigma, St. Louis, MO) were grown and maintained in high glucose Dulbecco's modified Eagle's media (DMEM, Millipore Sigma) supplemented with 10% fetal bovine serum (FBS, Millipore Sigma), 1% L-glutamine (Gibco, Grand Island, NY), 0.05% Hydrocortisone (Millipore Sigma), 1% antibiotic/antimycotic (Millipore Sigma). DOKs were starved in low-glucose serum-free DMEM (Gibco) overnight prior to experiments, followed by continued treatment in serum-free low glucose medium. Human-derived Leuk-1 cells (kindly provided by Dr. Hening Ren, University of Maryland, Baltimore) were grown and maintained in Keratinocyte Serum Free medium with growth factor supplement (Gibco) and 1% antibiotic/antimycotic. Leuk-1 cells were growth factor starved overnight, and treated continuously in the growth factor free medium. Human-derived spontaneously immortalized normal oral keratinocytes (NOK-SI) (kindly provided by Dr. Silvio Gutkind, University of California, San Diego) served as a normal, non-dysplastic oral keratinocyte control cell line. NOK-SI were grown, maintained, and treated under the same conditions as Leuk-1. Cells were cultured at 37 °C and 5% CO_2.

In specific experiments, cells were pre-treated for 2 h with inhibitors LY294002 (Cell Signaling, Danvers, MA), TVB-3166 (Millipore Sigma), cetuximab (obtained from the University of Maryland School of Medicine, Greenebaum Comprehensive Cancer Center Pharmacy, Baltimore, MD), AG1478 (Millipore Sigma), or rapamycin (Cell Signaling), followed by treatment with liquid (-)-nicotine (Millipore Sigma) for the indicated times.

2.2. FASN RNA interference

Short interfering RNA (siRNA) experiments were performed as previously described [27]. Briefly, DOK and Leuk-1 cells were plated at a density of 15,000 cells/cm^2. Cells were transfected with either 50 nM control, non-targeted scramble siRNA (Qiagen #1027280, Germantown, MD) or 100 nM FASN siRNA (Cell Signaling, #12613S) diluted in HiPerfect transfection reagent (Qiagen) following the manufacturer's recommendations. After 48 h, cells were cultured free of serum or growth factor overnight, and then treated with nicotine for 24 h.

2.3. Western blotting

After the indicated experiments, cells were lysed with M-PER mammalian protein extraction reagent (Thermo Scientific, Rockford, IL) supplemented with protease and phosphatase inhibitor cocktail (Thermo Scientific). Equal amounts of protein were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electrophoretically transferred onto polyvinylidene difluoride membranes, and immunodetection was performed by incubating membranes overnight at 4 °C with indicated antibodies as previously described [28]. The following Cell Signaling primary antibodies were used: rabbit monoclonal against FASN (#3180, 1:1000), rabbit polyclonal against phospho-Akt S473 (#9271, 1:1000), rabbit polyclonal against total Akt (#9272, 1:1000), rabbit monoclonal against phospho-EGFR Y1173 (#4407, 1:500), rabbit polyclonal against phospho-S6 S240/S244 (#2215, 1:1000), and rabbit monoclonal against total S6 (#2217, 1:1000). Rabbit polyclonal against total EGFR (#sc-03, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) and mouse monoclonal against β-Actin (#A5441, 1:20000, Millipore Sigma) were also used as primary antibodies. Peroxidase labeled goat anti-rabbit IgG (#5220-0458, 1:10000) and goat anti-mouse IgG (#5450-0011, 1:20000) were purchased from Seracare (Milford, MA) and used as secondary antibodies.

2.4. Wound closure migration assay

Cells were plated onto six-well plates in complete media at a density of 100,000 cells/cm^2 in order to reach near total confluency. The following day, cells were serum or growth factor starved overnight. This was followed by a 2-h pre-treatment with the indicated inhibitors. Next, the cell monolayer was uniformly scratched with a 200 μL pipet tip, followed by PBS washing thrice to remove cell debris. Treatment with either nicotine and/or continued specific inhibitor was then initiated for the indicated time period. Migrating cells toward the acellular area of the wells were photographed at a 10 × magnification with a digital camera attached to an Axiovert 100A microscope. The acellular gap was measured by area with ImageJ software (National Institutes of Health, Bethesda, MD) at the time of scratching (time 0) and at the final time point. Percentage of gap closure was calculated as ([Initial Area-Final Area]/Initial Area) x100.

2.5. FASN overexpression

To assess the effects of FASN in oral dysplastic keratinocytes, DOK cells were co-transfected with plasmids encoding human FASN (pCMV-SPORT6 vector containing FASN cDNA, catalog #MHS6278–202759913, Dharmacon, Lafayette, CO) and a puromycin resistance vector to select for FASN stably transformed cells (pPUR vector, catalog #631601, Takara, Mountain View, CA). Transfections were conducted by nucleofection according to the protocol provided by the manufactureer (Amaxa Biosystems, Köln, Germany). FASN stable overexpression was confirmed by clone selection of puromycin-resistant cells that showed high FASN expression by western blotting. As negative controls we used untransfected parental DOK cells and DOK cells transfected with pPUR vector only.