Original Article Local and systemic Curcumin C3 complex inhibits 4NQO-induced oral tumorigenesis via modulating FGF-2/FGFR-2 activation

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Abstract: Head and Neck Squamous cell carcinoma (HNSCC) can be characterized by synchronous tumors in the upper aerodigestive tract. Second primary tumors as a result of field cancerization are a significant problem amongst patients with risk factors for HNSCC, indicating a need for chemo preventive agents. We investigated the efficacy of local and systemic Curcumin C3 complex (C3); a purified mixture of Curcumin, bisdemethoxy Curcumin and demethoxy Curcumin as a chemo preventative agent in 4-nitroquinoline-1-oxide (4NQO)-induced tumorigenesis in mice. The effect of local C3 application was compared to C3 administered orally and in combination with systemic administration. C57BI/6 mice were administered 4NQO (50 µg/mI) in the drinking water for 16 weeks. At 12 weeks, mice were subjected to daily treatment with either vehicle (control), or 15 mg C3 complex by local delivery, gavage, or combined local and gavage for 28 days (16 week time point), and followed up to 22 weeks. Compared to local and oral systemic C3 administration, combination of local and systemic application significantly decreased multiplicity of 4NQO-induced preneoplastic and neoplastic lesions (p<0.05). Treatment with C3 correlated with a decrease in cell proliferation compared to the 4NQO group. Further, pre-treatment with C3 complex significantly attenuated 4NQO induced expression of basic fibroblast growth factor (FGF-2) and its cognate receptor FGFR-2, suggesting an important role of FGF-2/FGFR-2 axis in chemoprevention of HNSCC (p<0.05). Our findings suggest that a combination of local and systemic C3 complex could effectively target proliferation and inhibit 4NQO-induced tumorigenesis via modulation of the FGF-2/FGFR-2 axis as a mechanism for its efficacy.

Keywords: Oral squamous cell cancer, 4NQO, Curcumin C3 complex, FGFR-2

Introduction

Despite treatment advances, the overall survival rates from head and neck cancers have not improved significantly over the last three decades [1, 3]. About 90% of all head and neck cancers are squamous cell carcinomas (HNSCC) many of which involve the oral cavity and oropharynx. The annual incidence of head and neck cancers worldwide is greater than 550,000 cases with 300,000 deaths occurring each year. A vast majority of head and neck malignancies are tobacco-related [4, 5]. Cigarette smoking alone still accounts for 30% of all US cancer deaths, in spite of smoking prevalence reductions [3]. Tobacco use at HN-SCC diagnosis is a recognized risk factor for second primary tumors (SPTs), and current smokers are three times more likely than never-smokers to develop smoking-related SPT [6], indicating a need for chemo preventive agents among tobacco users. Development of local-regional recurrences from distant metastasis in advanced stage disease and second primaries in early stage disease further complicates treatment failure. Due to field cancerization, chronic exposure to carcinogens leads to development of several primary tumors in the upper aero digestive tract [2]. These secondary primary tumors are the most common cause of treatment failure and deaths among early-stage HNSCC patients [3]. To improve the outcome of such patients there is need for both local as well as systemic forms of chemoprevention that can afford protection in the upper aerodigestive areas. Our long-term goal is to prevent recurrences/second primaries of HNSCC. The incidence of second primaries in HNSCC patients can be as high as 22% within 5 years of treatment [3]. Thus, early stage intervention with non-toxic cancer chemo preventive agents could be a more practical alternative.

In our prior published dose response studies, we established that Curcumin C3 complex exhibited chemo preventive activity in a panel of HNSCC cell lines and in an in vivo model of 4-nitroguinoline-1-oxide (4NOO)-induced oral tumor formation. Compared to Curcumin, C3 complex is a purified mixture of Curcumin (76.07%); bisdemethoxy Curcumin (3.63%); and demethoxy Curcumin (20.28%). 4NQO is a synthetic, water soluble carcinogen that mimics the effects of chronic tobacco consumption in humans [4]. 4NQO induces oxidative stress, DNA adduct formation and A-G nucleotide resulting in histological and molecular alterations corresponding to those found in human oral carcinogenesis [5-9]. Curcumin is well established for its anticancer and chemopreventive efficacy in various preclinical models [10]. Curcumin is currently undergoing clinical trials for colon, skin, pancreatic, and hematologic cancers [11]. Recent evidence suggests an important role for Fibroblast growth factor (FGF) and its cognate receptors (FGFR's) in tumorigenesis [12]. FGFR belongs to the family of Receptor tyrosine kinases (RTKs). RTK's are transmembrane proteins with an extracellular ligand-binding domain, and a cytoplasmic tyrosine kinase domain. FGF family of RTK's is encoded by more than 22 genes that bind to and activate FGFR1-FGFR4 [13]. Aberrant FG-FR signaling, gene amplification, point mutation and chromosomal translocation have been reported during carcinogenesis [14]. Further, stronger expression of FGF-2 is reported in SCC than normal tissue [15]. FGF-2 is also known to promote cell invasion in oral squamous carcinoma via an autocrine mechanism. Increasing evidence suggests a vital role of FGF-2 and FGFR-2 in malignant transformation and cell proliferation [16].

In our recent published clinical trial results in HNSCC patients, treatment with C3 complex significantly decreased FGF-2 expression in po-

st treatment-biopsy samples (NCT01160302) [17]. Further, treatment with C3 significantly decreased serum levels of FGF-2 suggesting an important mechanism of C3-mediated effects *via* FGF-2 modulation. Accordingly, to further establish evidence for FGF-2/FGFR-2-dependent effects of C3, we utilized the 4NQO model of oral carcinogenesis. Additionally, we also compared the efficacy of local application of C3 to systemic administration and a combination of systemic and local C3 complex.

Methods

Reagents

4-Nitroquinoline-1-oxide (4NQO; Sigma-Aldrich, St Louis, MO) was added to drinking water to a final concentration of 50 ug/ml. Curcumin C3 complex (Sabinsa Corporation) was dissolved in corn oil and either administrated orally by gavaging or applied locally on the tongue in the form of a paste. OSC-19 cells used in this study was derived from a 61-year-old male with metastatic tongue cancer to the cervical lymph nodes.

Experimental animal model

All animal protocols were approved in advance by the Institutional Care and Use Committee at the LSU Health Shreveport in accordance with the policies and guidelines set forth by the Office of Laboratory Animal Welfare and the NIH. Female C57BI/6 (4-6 weeks) mice were obtained from Jackson laboratories. Mice were allowed to acclimatize in our animal facility for 2 weeks before the initiation of the experimental protocol. Forty 4-6 weeks old C57BI/6 mice received continuous dosing (50 µg/mL) of the tobacco-derivative 4-nitroguinoline 1-oxide (4NQO) in their water bottles daily for twelve weeks. It is well established that in the oralspecific chemical carcinogenesis model [8], 4NQO delivered in the drinking water to wildtype C57BI/6 mice for 16 weeks induces tumors in 50% of the mice at 12 weeks of exposure and 100% with tumors at 22 weeks (6 weeks after completing 4NQO exposure) [17-19]. Therefore, for our current studies, mice were exposed to 4NQO for twelve weeks. Immediately following the 12-week carcinogen exposure, mice were divided into groups that received vehicle (control) or C3 complex (15 mg in 100 µl total volume oil) by p.o. gavage



Figure 1. Experimental protocol for Curcumin (C3)-induced chemoprevention in 4NQO-induced oral tumorigenesis in mice. A. C57BI/6J mice were administered 4NQO in drinking water for 16 weeks. At 12 weeks of 4NQO treatment, mice were randomly divided into 4 groups: Group 1 (4NQO only), Group 11 (4NQO + local C3 application), Group III (4NQO + Gavage C3) and Group IV (4NQO + Local C3 + Gavage C3. B. Effect of treatment on body weight over the course of 16 weeks. C. Whole tongue images of lesions observed in mice at the end of 22 weeks since the initiation of carcinogen exposure with 4NQO for 16 weeks together with C3 treatment for 4 weeks as indicated in the Materials and Methods section.

(systemic), local Curcumin delivery (15 mg C3 in oil painted on their oral mucosa), or a combination of gavage and local C3 complex daily during weeks 12-16. 4NQO administration in drinking water continued during week 12 through 16. 4NQO mice were observed and weighed daily until week 22 to determine if C3 complex, whether applied locally or systemically, prevents carcinogenesis. All mice were sacrificed at 22 weeks, and tongues were harvested.

Immunohistochemical assessment of oral tongue and tumors

At the end of the experimental study, tongues were harvested at necropsy and analyzed for gross lesions, photographed and then fixed in formalin and embedded in paraffin. Longitudinally cut tongues were stained for hematoxylin and eosin (H&E). Tissues were verified by our study pathologist for degree of hyperplasia, dysplasia and occurrence of exophytic tumors. 5 um thick sections were processed for histopathological and immunohistochemistry analysis (IHC). Sections were immunostained for Ki67 (Santa Cruz biotechnology, 1: 200) as a marker for cell proliferation, FGF-2 (Cell Signaling, 1:200) and FGFR-2 (Santa Cruz biotechnology, 1:200). IHC sections were developed using either ImmPACT DAB or ImmPACT Vector Red (Vector Labs) with hematoxylin counterstain. Scoring of staining intensity was performed by the study pathologist on a scale of 0-3 with 0 + (no staining), 1 +(weak staining), 2 + (moderate staining), 3 + (strong staining). Proliferation was measured by counting red stained cells in 3 randomly selected areas per slide at 400× magnification.

In vitro cell proliferation and western blot analysis

Cell proliferation was measured using BrdU Cell Proliferation ELISA Kit (Abcam). Briefly, 2,000 cells per well were seeded in duplicate onto 96-well plates in complete media at 37° C with 5% CO₂. Cells were synchronized overnight in FBS-free media. Cells were pretreated with either C3 (10 µmol/L) or Vehicle for 1 hr followed by stimulation with FGF-2 (10 ng/ ml). At the end of 48 h, cell proliferation was



Figure 2. A. Histological examination of oral lesions. A detailed histopathological examination of oral tumorigenesis in mice post 4NQO exposure and treatment with C3 complex was carried out. Tongue tissues were collected, formalin fixed and processed further for H&E staining. Representative H&E-stained images of the entire tongue epithelium showing histologic changes of the tongue epithelia: normal squamous mucosa (a), (b) squamous mucosa with severe dysplasia and foci of superficially invasive squamous cell carcinoma (c) squamous mucosa showing mild hyperplasia (d) squamous mucosa with papilloma (e) squamous mucosa with papillary dysplasia. (f) 4NQO + local C3 tongue sections exhibiting hyperkeratosis (HPK) along with papillary dysplasia and mild dysplasia (g) 4NQO + Systemic C3 group showing HPK along with papilloma and dysplastic lesion. (h) Mice treated with 4NQO + Local + Systemic C3 showing mild dysplasia with HPK. B. Effect of C3 complex on 4NQO-induced tongue lesions. Mice were treated as indicated and at the end of study total tongue lesions in each group were recorded. Data represents average number of lesions per mouse (multiplicity) ± SEM for n≥9 in each group. One way ANOVA followed by Tukeys post-hoc test shows a significant effect of local and combination treatment compared to 4NQO control. *Denotes significance compared to 4NQO group only. #denotes significance compared to oral C3 treatment.

| Treatment | Mice with Lesions (%) | Mice with Exophytic tumors (%) | Total lesions |
|----------------------|-----------------------------|--------------------------------------|------------------|
| 4NQO | 100 | 80 | 29 |
| Local C3 | 90 | 20 | 19 |
| Gavage C3 | 100 | 60 | 28 |
| Local C3 + Gavage C3 | 80 | 44.44 | 16 |

Table 1. Effect of C3 complex on 4NQO induced

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measured using BrdU Cell Proliferation ELISA Kit (Abcam). For detection of FGF-2, pFGFR-2 and FGFR-2, cells were treated as indicated followed by extraction of soluble proteins from the cells. Lysates were subjected to ELISA for FGF-2 (b-FGF, Abcam), pFGFR-2 (Abcam) and total FGFR-2 (Cell Signaling).

Statistical analysis

ANOVA followed by the Tukey t test were used to analyze the differences between groups after treatments. Data analysis was done with GraphPad Prism version 6.01 for Windows (GraphPad Software); *P* values of less than 0.05 were considered statistically significant. Two-tailed, unpaired t-tests were used to analyze the differences in tumor growth between experimental groups, as well as differences in Ki-67 expression and low- and high-grade dysplasia between treated and control groups.

Results

Curcumin inhibits 4NQO-induced lesions

Over the course of the study (Figure 1A), mice were monitored for adverse effects and body



Figure 3. Effect of C3 on cell proliferation in 4NQO-induced tumorigenesis. A. Representative photographs of Ki67 with hematoxylin counterstain sections at 10× from (a) 4NQO, (b) 4NQO + local C3, (c) 4NQO + Gavage C3 and (d) 4NQO + Local + Gavage C3 groups. B. Briefly, positive stained cell were counted in 3 randomly selected area per slide at a magnification of 200×. Brown cells indicate positive staining for Ki67. Data was expressed as percent positive cells. *Denotes significance compared to 4NQO group only with *p* values <0.05.

weight was measured daily without any differences observed between treated and control animals. However, there was a trend towards decrease in body weight following the 13th week onwards (Figure 1B). Figure 1C shows whole tongue tissue from mice in the experimental groups. Consistent with prior published studies, mice that were administered 4NQO in drinking water for 16 weeks exhibited hyperkeratosis, hyperplasia (mild to moderate), dysplasia (mild to moderate), papilloma's and invasive squamous cell carcinoma with multifocal lesions. Figure 2A shows representative images of the oral lesions from 4NQO, 4NQO + Local C3, 4NQO + Systemic C3 and 4NQO + Local + Systemic C3. As shown in Table 1, both 4NQO and 4NQO + Gavage C3 group had a 100% incidence of oral lesions (hyperplasia, dysplasia and/or papilloma) compared to 90 and 80 percent in 4NQO + local C3 and 4NQO + Local + Gavage C3 group respectively. Besides a difference in the incidence of tumor development in the groups, there was also a striking difference in lesion multiplicity for the combination of local C3 and Systemic C3 group. At 16 weeks, mice in 4NQO and 4NQO + Gavage group showed 3.1 and 2.8 lesions/mouse respectively (Figure 2B). Local application of C3 exhibited a trend towards decrease in multiplicity with 2.1 lesions/mouse but did not achieve significance. Interestingly, combination of local and systemic C3 showed a significant decrease in tumor multiplicity with 1.6 lesions/ mouse (P<0.05). Further, the incidence of exophytic tumors in the local delivery C3 group (2/10, P<0.01) and the combination group (4/9, P<0.05)was statistically significant from the control group (8/9) and gavage delivery (6/10) (Table **1**).

Effect of Curcumin C3 complex on epithelial hyper proliferation

In our previous published studies, C3 complex inhibited cell proliferation in a wide range of HNSCC cell lines including SCC40, FaDu, PCI15a and SCC066. Accordingly, we sought to determine the mechanism for C3-mediated inhibition of 4NQO-induced epithelial proliferation. Tongue sections were subjected to Ki67 nuclear staining, a surrogate marker for cell proliferation. Cells that were positive for Ki67 exhibited distinct nuclear staining (**Figure 3A**). Treatment with local, systemic C3 and combination of local and systemic complex significantly inhibited 4NQO-induced proliferation in epithelial cells (P<0.05) (**Figure 3B**). However,



Figure 4. Effect of C3 complex on FGF-2/FGFR-2 signaling. (A) Cells were pre-treated with C3 complex for 1 h followed by exposure to FGF-2 (20 ng/ ml) and proliferation was assessed at 48 h using BrdU assay. Data represents mean \pm SEM for n=3 experiments. (*P<0.05) compared to Vehicle group and # denotes significance compared to FGF-2 stimulated group. C3 inhibits FGF-2 expression (B) and FGFR-2 (C) activation. FGF2 and pFGFR-2 expression levels were measured using ELISA kit (Cell Signaling). Data was normalized to the amount of protein used for the assay * and # denotes significance compared to NOK and OSC-19 vehicle treated group respectively. (D) To assess the effects of C3 on FGFR-2 expression, OSC-19 cells were either treated with Vehicle or Curcumin. At the end of 24 h, protein lysates were subjected to an ELISA for FGFR-2 detection. Data represents mean \pm SEM for n=3 experiments. *Denotes significance compared to OSC-19 cells

local and local with systemic groups shows higher efficacy in inhibiting cell proliferation compared to the group receiving only systemic treatment (P<0.01).

Effect of Curcumin C3 complex on FGF-2/ FGFR-2 activation

OSC19 cells were synchronized overnight in media containing 0.1% FBS. Cells were pretreated with either C3 complex or vehicle followed by stimulation with FGF-2 for 48 h. Pretreatment with C3 complex significantly attenuated FGF-2-induced OSC19 cell proliferation (Figure 4A). We also determined the effect of C3 on basal FGF-2 levels in OSC19 cells. As shown in Figure 4B, OSC19 express significantly higher levels of FGF-2 compared to NOK cells. Treatment with C3 complex for 24 h significantly decreased FGF-2 levels. It is well established that FGF-2 binds to FGFR-2 and induces autocrine signaling network in HNSCC. Accordingly, we investigated the effect of C3 on FGFR-2 expression in OSC19 cells. As shown in Figure

4C, compared to normal oral keratinocytes, OSC19 cells exhibited more than 5 fold increased expression of pFGFR-2. Treatment with C3 complex for 24 h significantly inhibited basal expression of pFGFR-2 in OSC19 cells. Interestingly, treatment with C3 complex decreased basal levels of FGFR-2 expression in these cells (Figure 4D). In an effort to identify the role of FGF-2/ FGFR-2 mechanism for C3mediated effects in our 4NQO model, immunohistochemistry for FGFR-2 and FGF-2 were performed. Overall, tissue from the 4NQO only group demonstrated strong cytoplasmic staining for FGF-2 and FGFR-2 (Figure 5A, 5B). Interestingly, local, systemic as well as the combination of local and systemic Curcumin C3 complex significantly decreased 4NQO induced FGF-2 expression (Figure 5B, P<0.05). However, only local and local plus systemic delivery C3 groups exhi-

bited significant inhibition of 4NQO-induced FGFR-2 expression (**Figure 5C** and **5D**, P<0.01). Thus, the effect of C3 complex on FGF-2 and FGFR-2 *in vivo* compliments its effects *in vitro*.

Conclusion

In spite of recent advances in detection, prevention, and treatment of oral squamous cell carcinoma (OSCC), the overall 5-year survival for OSCC still remains low [20]. Field cancerization leads to development of recurrences and second primaries that complicates the treatment strategy for OSCC [21]. Thus, it is critical to use primary and secondary prevention strategies along with early detection to improve long-term outcomes. During oral examination, clinicians can readily identify early stage premalignant lesions that have the potential to progress to advanced squamous cell carcinoma. Thus, treatment strategies that can potentially arrest the transformation or control the development of malignant lesions carries significant clinical relevance. Curcumin C3 com-



Figure 5. C3 inhibits FGF2 and FGFR-2 in 4-NQO model of OSCC. Tongue tissues were immunohistochemically stained for (A) FGF-2 and (C) FGFR-2. IHC sections were developed using ImmPACT Vector Red (Vector Labs). Staining intensity was quantified by a study pathologist on a scale of 0-3 as indicated in the materials and methods section. (B) Demonstrates all treatment groups showed significant reduction in 4NQO-induced FGF-2 expression. Upon quantification for (D) FGFR-2 staining only local and local + Systemic groups showed significant decrease in 4NQO induced FGFR-2 expression. *P<0.01.

plex is well established for its safety and efficacy in a large number of clinical trials. Further, its efficacy in prevention of tobacco induced HNSCC was demonstrated in preclinical studies [4, 22]. In this regard, our results demonstrate that local application of C3 complex on the mouse tongue and combination of local and systemic C3 afford protection against 4NQO-induced oral tumorigenesis. We have previously established the safety and efficacy of Curcumin C3 in clinical trials which could potentially expedite the use of C3 as treatment for prolonged periods and long term compliance (NCT01160302). Depending on the location, certain tumors of the oropharynx such as the soft palate that is known to have significant field cancerization and the larynx might be inaccessible for local application [23]. Thus using a combination of systemic and local C3 could potentially ensure drug deliverability at the site of tumor growth. Although there are several studies documenting the chemo preventive efficacy of Curcumin, this is the first study assessing the combination of oral and local Curcuminoid combination on 4NQO-induced OSCC.

Recent studies have highlighted the importance of FGF and FGFR signaling in a wide variety of human cancers including prostate, thyroid, urinary bladder, lung and head and neck [24]. In well differentiated tumors, FGF-2 is significantly upregulated suggesting an important role of FGF-2 in dysplasia and oral squamous cell carcinoma [15, 25]. Additionally, FGFR-2 is increased in OSCC, while inhibition leads to apoptosis [26]. In our previous published studies in patients with HNSCC, using immunohistochemical analysis we showed that as the grade of dysplasia increased, the percentage of cases expressing FGF-2 also increased [27].

Further increased expression, somatic mutations and gene amplification of FGF and FGFR has been observed suggesting an important role of FGF-2/FGFR-axis in oncogenesis. FGFR inhibitors are currently under phase 2 clinical trials for treatment of small cell lung carcinoma and breast cancer [28].

In our prior published studies, exposure of HNSCC cell lines to nicotine induced FGF-2 expression [4]. These results were consistent with other published studies demonstrating the importance of FGF-2 in HNSCC [16]. Recent studies from our lab have also highlighted the importance of FGF-2 and its cognate receptor FGFR-2 in C3-mediated effects. In a recent phase 0 clinical trial at our institute evaluating the efficacy of Curcumin C3 in HNSCC patients, 7 out of 11 patients showed a significant decrease in FGF-2 expression compared to their respective pre-treatment biopsies. Further, there was a significant decrease in serum FGF-2 levels post C3 administration. Therefore we hypothesized that FGF-2 signaling mechanism could play an important role in C3-mediated effects on 4NOO-induced oral tumorigenesis. Our studies determined that local application of C3 and the combination of local and systemic C3 significantly inhibited 4NQO-induced FGF-2 and FGFR-2 expression in 4NQO exposed tongue epithelium highlighting the importance of FGF-2/FGFR-2 signaling in Curcumin-mediated effects. The exact mechanism for FGF-2/FGFR-2 activation in our study needs further investigation as FGFR-2 can be activated by both ligand-dependent and independent mechanisms. Studies conducted in our lab using an UVB-induced skin carcinogenesis model suggest that FGFR-2 modulates mTORC1 and mTORC2 leading to activation of pS6K and pAKT which are hyper activated in both precancerous and cancerous dysplasia and advanced HNSCC (unpublished data).

The increase in 4NQO-induced FGF-2 expression could potentially stimulate HNSCC cell proliferation. Accordingly, we sought to determine the anti-proliferative effect of Curcumin C3 complex on FGF-2-induced epithelial cell proliferation. C3 was effective in blocking 4NQOinduced epithelial hyper proliferation both in local and systemic application and exhibited growth inhibitory effects potentially via inhibition of the FGF-2/FGFR-2 axis. Our studies for the first time revealed that a combination of local and systemic C3 inhibit 4NQO-induced tumor promotion and progression. Overall, C3 appears to be an ideal chemo preventive agent in both premalignant and malignant tumor management of HNSCC. We show here that Curcumin inhibits the earlier stages of tumor development in mice indicating Curcumin's potential role as a chemopreventive agent. The significant difference in the local Curcumin treatment group compared to control demonstrates Curcumin has growth inhibitory effects and prevents tumor formation in an oral carcinogen-induced model. The dose and delivery method required for bioactive food compounds is an important question and local Curcumin delivery was required to prevent tumor formation. This preferential inhibition of tumor formation in HNSCC by prolonged local contact could be useful in the design of clinical trials with Curcumin.

Disclosure of conflict of interest

None.

Abbreviations

C3, Curcumin C3 complex; 4NQO, 4-nitroquinoline-1-oxide; OSCC, Oral Squamous Cell Carcinoma; HPK, Hyperkeratosis; FGF, Fibroblast Growth Factor; FGF2, Fibroblast Growth Factor 2; FGFR, Fibroblast Growth Factor Receptor; FGFR-2, Fibroblast Growth Factor Receptor 2.

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