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ORIGINAL RESEARCH

The Effects of 5-fluorouracil Alone and in Combination with 13-cis Retinoic Acid and Vitamin D_3 on Human Oral Squamous Cell Carcinoma Lines

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ABSTRACT

Aim: Oral squamous cell carcinoma (OSCC) is responsible for about 90% of oral malignancies and its incidence is increasing. Despite various treatment protocols, survival rate of OSCC is low. Chemotherapy that is used for treating this carcinoma in advanced stages is systemic therapy that destroys carcinogenic cells, and controls tumor metastasis. Chemotherapy is very toxic and has limitations, especially for patients in advanced stages. Considering positive effects of retinoid and vitamin D_3 derivatives in treating some carcinomas, we decided to evaluate the effect of combination of these drugs on OSCC. In this study the effects of combination of 5-fluorouracil, 13-cis retinoic acid and vitamin D_3 on cultured cell of OSCC have been evaluated.

Materials and methods: OSCC cells were cultured in culture media and different concentration of 5-fluorouracil, 13-cis retinoic acid and vitamin D_3 were added to cultured cell as separately and in combinations. The effect of treatment on cell proliferation and induction of apoptosis were evaluated by MTT and TUNEL assays respectively.

Results: Combination of 5-fluorouracil and 13- cis retinoic acid had the highest inhibitory effect on SCC cell proliferation. Combination of two drugs had more apoptotic effect than each of them separately, and combination of three drugs had more effect than combination of two drugs.

Conclusion: Because combination of drugs had more inhibitory effect on cell proliferation than one of them and combination of three drugs had the most apoptotic effect than one of these drugs separately, these drugs may have synergic effect on OSCC.

Clinical significance: Combination of three drugs has more inhibitory effect on cell proliferation and apoptotic effect than one of these drugs.

Keywords: Oral, Squamous cell carcinoma, Retinoic acid, Vitamin D, 5-fluorouracil.

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INTRODUCTION

Squamous cell carcinoma (SCC) is over 90% of oral malignancies and its incidence is increasing worldwide. Ninety five percent of patients are above 40 years old and mean age of patients in diagnostic time is 60 years old.[1]

The rate of recurrence is high in head and neck SCC and this cancer is associated with primary and secondary malignancies.[2] Proper treatment is very important, because mostly, there is not any secondary chance for treatment of this cancer. Because of limitation of chemotherapy in advanced cancers, surgery and radiotherapy have been introduced as golden standard for treating OSCC. It has shown that SCC can successfully be treated by surgery or radiotherapy in I or II stage.[3] But early diagnosis of SCC has not changed in recent three decades and 2/3 of patients are in progressive stages (III, IV stages) in diagnosis time.[4] Chemotherapy that kills the cancerous cells is used for treatment of this tumor in progressive stages.[3] Chemotherapy is a systemic treatment that kills the cancerous cells by different mechanisms such as induction of apoptosis. There are more than 50 different types of chemotherapy drugs which some of them are used separately but mostly are used together for treatment. Because of unknown mechanism, most of oral cancers show poor response to chemotherapy. Despite different treatment strategies—from palliative treatments to complete resection—for clinical management of oral cancer, survival rate of OSCC is low and researchers try to find new treatment protocols for oral cancer.

Nowadays, different cytotoxic drugs such as 5-fluorouracil is used for oral cancer treatment. Retinoic acids and vitamin D

analogs have been used in treatment of different cancers but a few studies have been done on OSCC. Because of positive effects of vitamin D and retinoic acids analogs in treating of cancers, we decided to compare the effect of vitamin D, retinoic acid and 5-fluorouracil with 5-fluorouracil alone on cultured oral SCC cells and evaluate their probably synergism effects. The results of this study can be useful in making of composition drugs for oral SCC.

MATERIALS AND METHODS

Cell Culture

Human oral squamous cell carcinoma lines (C152) was obtained from Pasteur Institute of Iran. The cells were cultured in PRMI-1640 with 10% inactivated fetal calf serum (FCS) containing L-glutamine (2 mm) and penicillin/streptomycin (50 µg/ml, 50 µg/ml) at 37°C in 5% CO_2 atmosphere.

MTT Assay

For evaluation of effects of testing drugs on cell proliferation, MTT-based colorimetric assay was used. SCC cells (200000cell/ml) were plated in 96 well flat-bottomed plates and incubated at 37 for 24 hours. After addition of 5-fluorouracil (7.7 µm and 385 µm); 13-cis retinoic acid (10 µm and 50 µm) and vitamin D_3 (10 µm and 20 µm) as alone and double and triple combinations, the plates were incubated at 37°C for 24 hours. After that, the media was removed and 200 µl new cultural media and 50 µl of dimethylthiazol-diphenyl tetrazolium bromide (MTT) (2 mg/ml) were added and the plates were incubated at 37°C for 3 hours. Then, the supernatant was removed and 200 µm DMSO and 25 µl Sorensen glycine buffer was added in to the microplate wells. After 5 minutes shaking, the absorbance of wells was read at 570 nm using a spectrophotometer. The inhibition of cell proliferation by drugs was compared with control group.[5,6]

TUNEL Assay

Induction of apoptosis by tested drugs on SCC cell line were determined using TUNEL assay (*In Situ* Cell Death Detection Kit, Roche Diagnostics Corporation) following the manufacturer's instructions.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) is a common method for detecting DNA fragmentation that results from apoptotic signaling cascade. The SCC cells (200000 cell/ml) were cultures on coverslips in petri dishes at 37°C for 24 hours. Culture media was changed and 5-FU (Concentrations 7.7 µm and 385 µm); 13- cis RA (Concentrations 10 µm and 50 µm) and vitamin D_3 (Concentrations 10 µm and 20 µm) as alone and double and triple combination were added to the plates and incubated for 24 hours. Then the culture media was removed and the cells on the coverslips were washed with PBS (phosphate- buffered saline) and fixed with glutaraldehyde solution. The TUNEL reaction was carried out after treatment with proteinase K. The coverslips were incubated with a mix solution composed of the enzyme terminal eoxynucleotidyl transferase (TdT; 1 µl/200 µl of mix solution), bovine serum albumin (1 mg/ml), and biotin 16-dUTP (1 nmol/50 µl of mix solution) in TdT buffer. The coverslips was then placed in a solution of saline citrate for 15 minutes at room temperature, rinsed with distilled water followed by tris(hydroxymethyl) aminomethane (Tris)-buffered saline (TBS) for 1 to 2 minutes, and then incubated with 3% bovine serum albumin at room temperature. The coverslips were rinsed again with distilled water and with TBS and incubated with 3% hydrogen peroxide in TBS to block endogenous peroxidase activity. The labeled DNA fragments were detected with peroxidase-conjugated antibody elicited against biotin. Diaminobenzidine was used as the substrate for peroxidase, yielding the characteristic brown color for nuclei. After rinsing in distilled water, coverslips were counterstained with methyl green. The apoptotic index was defined by the percentage of brown (dark) cells among the total number of cells in each sample. Twenty fields with 100 cells per field were randomly counted for each sample[7] (Elmagasli). The average of colored cells percents (brown cells) in comparison with control cells was evaluated. Counting was performed at ×400 magnification.

RESULTS

Effect of 5-fluorouracil, 13-cis Retinoic Acid and Vitamin D_3 on Proliferation of SCC Cells

The results of MTT assay revealed that that 385 µmol concentration of 5-fluorouracil inhibited the proliferation of SCC in treated group in comparison with control group. It means 5-fluorouracil in this concentration prevents proliferation of SCC cells. 13-cis retinoic acid inhibited proliferation of SCC cells at 50 µmol concentration whereas vitamin D_3 showed 50% inhibition in 20 µmol concentration (Table 1).

Combination of 5-fluorouracil and 13-cis retinoic acid had the highest inhibitory effect on SCC cell proliferation. Among combination of drugs, combination of 13-cis retinoic acid and vitamin D_3 had the least inhibitory effect on SCC cells proliferation (Table 2).

Table 1: Percent of cell proliferation in cultured cells of oral squamous cell carcinoma using MTT assay according to different concentrations of drugs

Drugs	*Vit D_3*	*Vit D_3*	*13-cis RA*	*13-cis RA*	*5-FU*	*5-FU*
Concentration (µm)	10 µm	20 µm	10 µm	50 µm	7.7 µm	385 µm
Proliferation	2.10	1.85	1.628	0.821	1.282	0.835

Table 2: Percent of cell proliferation in cultured cells of oral squamous cell carcinoma using MTT assay according to different drugs combination

Combination of drugs	*Without drugs*	*RA (10 µm) Vit D_3 (20 µm)*	*5-FU (385 µm), Vit D_3 (20 µm), RA (50 µm)*	*5-FU (385 µm) Vit D_3 (20 µm)*	*5-FU (385 µm) RA (50 µm)*
Proliferation	2.810	1.639	0.723	0.796	0.427

Effect of 5-fluorouracil, 13-cis Retinoic Acid and Vitamin D_3 on Apoptosis of SCC Cells

Percent of apoptosis was evaluated with TUNEL assay and demonstrated that 13-cis retinoic acid had the highest apoptosis induction effect followed by vitamin D_3 and 13-cis retinoic acid respectively.

Combination of 3 drugs induced the highest apoptosis induction effect by 87%. Combination of vitamin D_3 and 13-cis retinoic acid led to apoptosis induction by 70%. Among combination drugs, combination of 13-cis retinoic acid and 5-fluorouracil led to less apoptosis induction and combination of vitamin D_3 and 5-fluorouracil caused the least apoptosis induction.

DISCUSSION

Oral cancer is a major global threat for public health. In every year many new cases are diagnosed worldwide. Despite different treatment strategies, the great morbidity and mortality rates of this cancer have not reduced in recent decades.[8]

Second primary tumors and metastasis remain significant causes of morbidity and mortality.[9]

Research for finding new treatment protocols continues.

In this study, effect of vitamin D_3 and 13-cis retinoic acid and 5-fluorouracil and combination of them was evaluated on cultured oral SCC cells. The results revealed that the most apoptosis induction percent earned with using combination of 13-cis retinoic acid, vitamin D_3 and 5-fluorouracil.

The most inhibitory effect on cell proliferation was related to combination of 13-cis retinoic acid and 5-fluorouracil that was even more than combination of 3 drugs.

The results suggest addition of 13-cis retinoic acid and vitamin D_3 to 5-fluorouracil can increase the anticancer effect of 5-fluorouracil on oral SCC cells.

To our knowledge, some studies have been done about the effect of retinoid and vitamin D analogs on various cancers, but no research has been done about the combination of them with 5-fluorouracil -as an anticancer drug- on oral SCC cells.

One study on animal models showed metabolic and synthetic and natural analogs of vitamin A limit carcinogenesis in lung, head and neck.[10]

It should be considered that different retinoid analogs have various efficacy and plasma half-life, so can have different effects.

All-trans retinoic acid (ATRA) has greater affinity for retinoic acid receptors and may be more active than other retinoid analogs but its short plasma half- life may up-regulate its own metabolism.[9]

One study revealed that all-trans retinoic with dose of 150 mg/m^2/day every 8 hours is a tolerable dose for 1 year in patients with treated head and neck SCC.[9]

Animal study on anticancer effect of antisense oligonucleotides against hTR (As-ODN-hTR) combined with all-trans retinoic acid (ATRA) has been done. This research revealed that both As-ODN-hTR and ATRA can significantly inhibit tumor and the combination of them has a synergistic anticancer effect.[11]

One animal study revealed that the combination of 5-Aza-2'-deoxycytidine (DNA hypermethylation) and the low dose of all-trans retinoic acid was effective in reduction of oral cavity cancer.[12]

Anticancer agents with different mechanisms cause inhibitory effects.

Nowadays, new techniques, such as interventions with molecular-targeted agents and agent combinations are used in molecularly defined high-risk patients to decrease the consequences of oral cancer.[8] One study showed retinoic acid inhibits SCC cells growth without stopping DNA synthesis.[13] Another study demonstrated that treatment of human squamous cell carcinoma cells with acyclic retinoid (ACR) inhibits tumor growth by various mechanisms. It causes a decrease in cellular levels of the cyclin D1 protein and mRNA, TGF-_ mRNA and TGF-_ protein. Also, using

this agent inhibits activation of the TGF-_/EGFR pathway and increases cells in G0 to G1 and induces apoptosis.[14] Also, retinoid analogs can inhibit SCC with inhibition of PGE2 synthesis.[15]

It is confirmed that alterations in expression of retinoids receptor influence regulators of cell cycle and consequently, can affect carcinogenesis.[16]

Few studies with different results about effect of vitamin D on SCC have been done.

The mechanisms of the effects of vitamin D analogs include cell cycle arrest, induction of apoptosis, differentiation and modulation of growth factor-mediated signaling in tumor cells and antiproliferative and anti-angiogenesis effects.

1-alpha-25-dihydroxy vitamin D_3 increases cell cycle arrest and apoptosis.[17]

Furthermore, inhibition of angiogenesis plays a role in antitumor affect of active form of vitamin D.[18]

One animal study demonstrated that systemic treatment with 1-alpha, 25-dihydroxy vitamin D_3 inhibits carcinogenesis in the hamster buccal pouch model.[19]

Another study showed using of vitamin D can stop cancer cells cycle in tongue and laryngeal SCC.[20]

Arresting of cell cycle in G0, G1 phases due to up-regulation of p18 and p21, is one mechanism by which vitamin D_3 inhibits cell growth of head and neck SCC cell lines.[21]

Another study revealed that two low- calcemic vitamin D analogs had anticancer effects in the Murine SCC cells line were generated from spontaneous tumors of the C3H strain of mice. The vitamin D analogs modulated cell cycle regulators and induced apoptosis and subsequently inhibited SCC cell growth.[22]

Vitamin D stimulates biosynthesis of EGF receptor. Using of vitamin D_3 for long time can induce regulation of proliferation and differentiation and can be effective in cancer treatment.[23]

In contrast, another study revealed SCC cells cannot respond to vitamin D_3 for more differentiation. There is an abnormality in control of genes expression by $1,25(OH)_2D_3$ in keratinocytes.[24]

Some studies have been done about vitamin D intake and risk of development of cancers.

Although, one study done in Italy revealed that about esophageal cancer, there is an inverse relation with vitamin D intake, especially among persons aged above 60 years, the opposite is true for oral and pharyngeal cancers.[25] A cross-sectional study done in China, on a population at high risk for development of esophageal SCC showed a direct relation between dysplasia and higher median serum 25-hydroxy vitamin D concentrations.[26]

Present study showed effect of antiproliferative and apoptosis induction of 13-cis retinoic acid, vitamin D_3 and 5-fluorouracil combination, too.

Other studies revealed synergism effect of combination of retinoid or vitamin D analogs with routine chemotherapy drugs.

Lingen et al treated animals bearing human head and neck SCC tumor cells with a combination of retinole acid and IFN-α A/D at doses that were ineffective when used alone. The results showed dramatic decreases in tumor growth and tumor angiogenesis suggesting combination of antiangiogenic agents may be effective strategy for designing newer chemoprevention protocols against SCC.[27]

Also, another study on nasopharyngeal carcinoma and squamous cell carcinoma cell lines, and head and neck cancer specimens- using MTT assay- demonstrated that combination of interferon α and vitamin A, has cell- growth inhibitory effect more than one of them, alone.[28]

Zhang et al in 2006 evaluated effect of 13-cis retinoic acid, interferon α and α-tocopherol, as alone and 2 and 3 drug combination in head and neck SCC and showed that combination of 3 drugs inhibits cell growth and inducts more apoptosis than any of these agents that can show cooperative or synergism effect of these drugs.[29]

Addition of retinoic acid to 5-fluorouracil or cisplatin increases the treatment effectiveness and confirmed that retinoic acid has synergism effect with chemotherapeutic drugs. It seems that retinoic acid increases sensitivity of SCC cells to chemotherapeutic drugs.[13]

Pretreatment with a vitamin D analog (Ro23-7553) in a SCC model system potentiates killing of SCC cells by cisplatin via cell cycle arresting, *in vitro* and *in vivo*.[30]

Researchers suggest vitamin D_3 promotes p73 induction. Because of p73 that is one of the p53 family members regulates apoptosis, 1, 25 D_3 sensitizes SCC cells to cisplatin-mediated apoptosis.[31]

These results showing 1,25 D_3 and cisplatin have synergistic effect on inhibition of SCC cells, is similar to our study results.

About synergism effect of retinoid analogs and cisplatin, one study assessed the effect of 9-cis retinoic acid on cisplatin cytotoxicity in a human oral squamous carcinoma xenograft model of the mice. It showed that 9-cis RA changes squamous cell carcinoma phenotypes via suppressing cell differentiation and can be effective in tumors with increased numbers of basal cells. These results revealed that 9-cis RA increases tumor sensitivity to cisplatin.[32]

These results are similar to present results showing probably synergism effect of retinoid analogs and anti-cancer drugs.

Beside increase in antitumor effect, decrease in cell toxicity is important in studies on anticancer agents. The researchers suggested combination of 13-cis retinoic acid and vitamin D_3 can be less toxic and more effective in treatment of head and neck carcinoma.[33]

Therapy with many drugs such as cisplatin that is commonly used for treatment of head and neck tumors, frequently fails due to development of resistance or toxicities associated with these agents. Therefore, finding of new strategies for treating oral cancers is essential.

Combination of some drugs may be more effective and less toxic than one of them.

It is suggested that more studies designed on animal models for evaluation of effectiveness and side effects of these drugs. If other studies confirmed the similar results in other animal studies and clinical trials, this combination can be used as supplementary element in treatment of oral squamous cell carcinoma.

CONCLUSION

Combination of 13-cis retinoic acid and 5-fluorouracil had the most inhibition effect on proliferation of oral SCC cells in comparison with 5-fluorouracil and another combination drugs.

About apoptosis induction, triple combination had better effect in comparison with double combination and double combination were more effective than one of them.

CLINICAL SIGNIFICANCE

Combination of three drugs has more inhibitory effect on cell proliferation and apoptotic effect than one of these drugs. The results can be indicating probably synergism effect of these agents on OSCC cells.

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