Acta Odontologica Scandinavica, 2012; 70: 241–245

informa
healthcare

ORIGINAL ARTICLE

Poly I:C inhibits cell proliferation and enhances the growth inhibitory effect of paclitaxel in oral sqaumous cell carcinoma

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Abstract

Objective. Toll-like receptors (TLR) signaling has dual effect of promoting tumor progression and anti-cancer property. This study was designed to determine the effect of polyinosinic-polycytidilic acid (poly I:C), a TLR3 agonist, on the proliferation of oral cancer cells. ***Materials and methods:*** Human oral squamous cell carcinoma cell lines, YD-10B and YD-8, were used. TLRs expression was examined by RT-PCR and IL-8 production by poly I:C was examined by ELISA. Cell proliferation was determined by MTT assay. Flow cytometry and Western blot analysis were performed to determine the molecular mechanism of poly I:C-induced cell death. ***Results.*** TLR3 was functionally expressed in YD-10B and YD-8 cells. Treatment of poly I:C inhibited the cell growth in a dose-dependent manner. Flow cytometry and Western blot analysis revealed that poly I:C induced apoptosis via a mitochondria-dependent pathway. In addition, combination treatment with poly I:C and paclitaxel more significantly inhibited cell proliferation compared with poly I:C or paclitaxel alone. ***Conclusions.*** Poly I:C effectively inhibits oral cancer cell proliferation and can be considered as a candidate to improve the inhibitory effect of anti-cancer drugs.

Key Words: *Toll-like receptor 3 (TLR3), Poly I:C, oral sqaumous cell carcinoma, apoptosis, paclitaxel*

Introduction

Innate immune response is the first line of host defense against microbial infections including bacteria, viruses, parasites and fungi. Common molecular structures of micro-organisms are recognized by so-called pattern recognition receptors (PRRs), which initiate various innate immune mechanisms. Among PRRs, Toll-like receptors (TLRs) are the best known and studied. TLRs sense various microbial molecules such as lipopolysaccharide (LPS) (TLR4), lipoprotein (TLR2), flagellin (TLR5) and viral nucleic acids (TLR3, 7 and 9), which result in the activation of downstream signalings such as nuclear factor kappa B (NF-κB), mitogen-activated protein kinases (MAPKs) or interferon regulatory factors (IRFs) [1,2].

TLR agonists have been evaluated for the treatment of cancers. Recent studies revealed that systemic or local application of TLR agonists enhances innate immunity, T-cell immunity and cytotoxic antibody function and induces apoptosis in TLR-positive tumors [3–5]. Among TLRs, TLR3 recognizes viral double stranded RNA (dsRNA) and activates NF-κB and IRF3 through TIR domain-containing adaptor protein inducing an interferon β (TRIF)-dependent pathway. There is controversy for the effect of TLR3 on tumor cell proliferation. Several studies revealed that TLR3 activation by polyinosinic-polycytidilic acid (poly I:C), a synthetic dsRNA, directly induced apoptosis in various cancer cells [6,7]. In contrast, TLR3 inhibition using siRNA led to inhibition of cell growth and poly I:C treatment promoted cell proliferation of head and neck cancer, suggesting TLR3 is involved in promoting tumor growth in head and neck cancers [8].

Oral squamous cell carcinoma (OSCC), one of the top 10 most commonly occurring cancers worldwide, has a high mortality rate. In spite of recent improvements and advances in medicine such as surgery, radiation and chemotherapy, the 5-year survival rate for oral cancer patients has remained

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(Received 27 April 2011; accepted 3 August 2011)

ISSN 0001-6357 print/ISSN 1502-3850 online
DOI: 10.3109/00016357.2011.640278

at 50% over the past five decades [9,10]. Several clinical applications of surgery, radiation and chemotherapy are standard treatments for early oral cancer, which result in effective tumor control. However, many severe side-effects and/or toxicity appear in patients receiving those treatments [11]. Therefore, an alternative therapeutic strategy has been demanded.

Recently, we examined TLR expression and function on a human OSCC cell line, YD-10B cells [12]. In the study, we revealed that YD-10B cells functionally expressed various TLRs including TLR3 [12]. However, the effect of TLR3 on the proliferation of OSCC cells was not known. In this study, we examined the effect of poly I:C, a TLR3 agonist, on the proliferation of human OSCC cell lines, YD-10B and YD-8 cells.

Materials and methods

Cell lines and reagents

Human OSCC cell lines, YD-10B and YD-8, were purchased from Korea Cell Line Bank (KCLB) (Seoul, Korea). The cells were incubated in RPMI medium including 10% fetal bovine serum and 1× Penicillin streptomycin at 37°C containing 5% CO_2. Polyinosinic-polycytidilic acid (poly I:C) was purchased from Invivogen (San Diego, CA). In all experiments, Fugene HD transfection reagent (Roche, Indianapolis, IN) was used to improve cytosolic entry of poly I:C.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from each cell using a Qiagen RNeasy Mini kit (Valencia, CA). One microgram of total RNA was reverse-transcribed into cDNA and PCR was performed using the Superscript™ One-step RT-PCR with platinum® Taq kit (Invitrogen, Carlsbad, CA). The following primer sets were used.

- TLR3, F: 5′-GTGCCAGAAACTTCCCATGT-3′
- R: 5′-CTTCCAATTGCGTGAAAACA-3′
- IFN-β, F: 5′-GATTCATCTAGCACTGGCTGG-3′
- R: 5′-CTTCAGGTAATGCAGAATCC-3′
- GAPDH, F: 5′-CCAAGGTCATCCATGACAACTTTG-3′
- R: 5′-GTCATACCAGGAAATGAGCTTGACA-3′

PCR condition consists of one cycle of 94°C for 3 min, 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s and one cycle of 72°C for 10 min. PCR products were then electrophoresed on a 1.5% agarose gel and visualized using a gel documentation system.

Enzyme-linked immunosorbent assay (ELISA)

The cells were plated in 48 well plates at 2×10^4 cells/well overnight and treated with various doses of poly I:C for an additional 24 h. Culture media was harvested for measurement of IL-8 concentration. The ELISA was performed according to the manufacturer's instructions (R & D Systems, Minneapolis, MN). The absorbance of the reaction solution was measured at 450 nm on a Microplate Autoreader ELISA (Bio-Tek Instruments Inc., Winooski, VT).

MTT assay

The cells (2×10^4 cells/well in 48 well plates) were incubated with various doses of poly I:C for 24 h. The cells were washed twice with ice-cold phosphate buffered saline (PBS, pH 7.4) and 0.5 ml of cell culture medium and 50 μl of 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide reagent (5 mg/ml in PBS) were added and incubated for 3 h at 37°C. The media was then removed and 250 μl of acid-isopropanol (0.04 mol/L HCl in isopropanol) were added. The optical density (OD) was then measured by a Microplate Autoreader ELISA (Bio-Tek Instruments Inc., Winooski, VT) at 570 nm wavelength.

Flow cytometry

After treated with poly I:C for 24 h, the cells were stained with Alexa Fluor 488 Annexin V and propidium iodide (Vybrant Apoptosis Assay kit, Molecular Probes), according to the manufacturer's protocol. After being incubated for 15 min in the darkness at room temperature, the 1×10^4 cells per sample were analyzed immediately using a Cell Llab QuantaTM SC flow cytometer (Beckman Coulter Inc, Miami, FL) and software.

Western blot analysis

After being treated with poly I:C for 24 h, the cells were lysed in lysis buffer (137 mM NaCl, 15 mM EGTA, 0.1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 25 mM MOPS, 100 μM phenylmethylsulfonyl fluoride and 20 μM leupeptin, adjusted to pH 7.2). The total protein (40–50 μg per lane) was resolved by 7.5–15% SDS-PAGE and transferred onto PVDF membranes. After blocking in TBS (20 mmol/L Tris, 137 mmol/L NaCl, 1 g/L, pH 7.6) with 5% skim milk for 2 h at room temperature, the membranes were incubated with primary antibodies at 4°C overnight. Antibodies for Bcl-2, Poly (ADP-ribose) polymerase (PARP) and actin were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for cytochrome C and cleaved caspase-7/-9 were from Cell Signaling (Danvers,

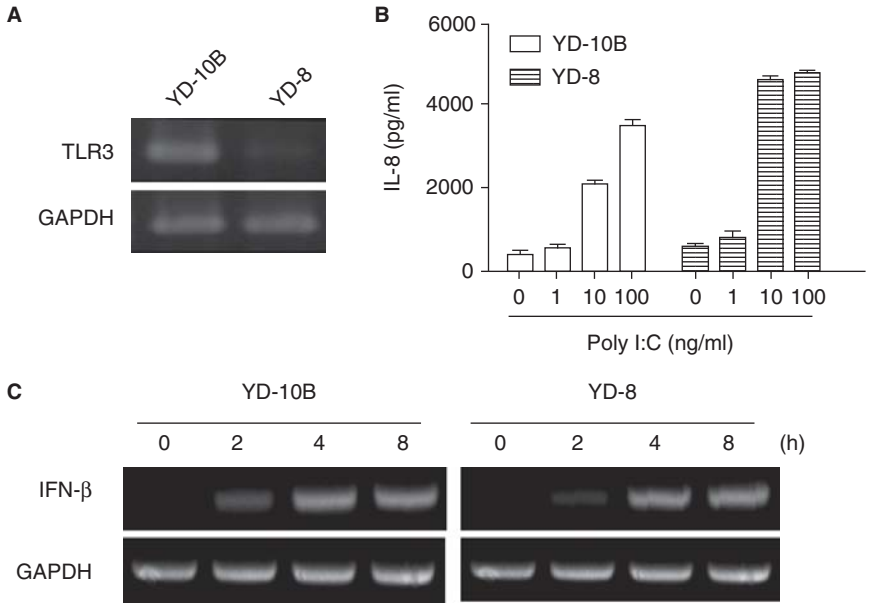

Figure 1. Functional expression of TLR3 in human OSCC cell lines, YD-10B and YD-8 cells. mRNA expression of TLR3 and GAPDH was examined by RT-PCR (A). The cells (2×10^4 cells/well in 48 well plates) were incubated at the presence of various doses of Poly I:C for 24 h. Culture supernatant was collected and IL-8 production was measured by commercial ELISA kit (B). The cells were treated with 100 ng/ml of poly I:C and RNA was extracted at 0, 2, 4 and 8 h after treatment. The gene expression of IFN-β was examined by RT-PCR (C).

MA). The membranes were then washed three times with a 0.05% tween-TBS (TBS-T), followed by incubation for 1 h with secondary antibodies (1:5000; Santa Cruz Biotechnology) at room temperature. Finally, the membranes were visualized using the West ZOL PLUS detection reagent in the LAS-1000 (Fujifilm, Japan).

Statistical analysis

The differences in mean values among different groups were tested and the values were expressed as mean ± SD. All of the statistical calculations were calculated by one-way ANOVA followed by Bonferroni post-hoc test for multi-group comparisons (StatView 5.0; SAS Institute, Cary, NC). Statistical significance is indicated in the figure legends.

Results and discussion

Diverse combination therapy targeting multiple pathways has been used for cancer treatment. Combined with anti-cancer drugs, poly I:C (a synthetic TLR3 agonist) has been used for treatment of various cancers including colon, breast and ovary [13–15]. In addition to direct apoptosis on tumor cells, poly I:C is proved to enhance drug-induced apoptosis of tumor cells through a TLR3-dependent pathway [16]. In contrast, a few studies claimed that TLR3 is involved in promoting cell proliferation, even though there is insufficient evidence [8,17]. These findings indicate that TLR3 exhibits a dual effect on cell proliferation, which may be cell type specific. Until now, it is unknown whether and how dsRNA regulate cell proliferation in oral cancer cells. In this study, we first examined mRNA expression of TLR3 in two human OSCC cell lines, YD-10B and YD-8 cells, by RT-PCR. As shown in Figure 1A, the gene of TLR3 was detectable in the cell lines, although the expression level was weak in YD-8 cells. Sensing of dsRNA by TLR3 leads to the activation of NF-κB or IRF3 via a TRIF-dependent pathway, which results in the production of pro-inflammatory cytokines or anti-viral type I interferon, respectively [1,2]. To examine whether TLR3 expressed in YD-10B and YD-8 cells can respond to its agonist, IL-8 production and IFN-β expression by poly I:C were determined by ELISA and RT-PCR, respectively. Poly I:C increased IL-8 production in both cell lines in a dose-dependent manner (Figure 1B). In addition, the gene expression of IFN-β was increased by poly I:C from 2 h after

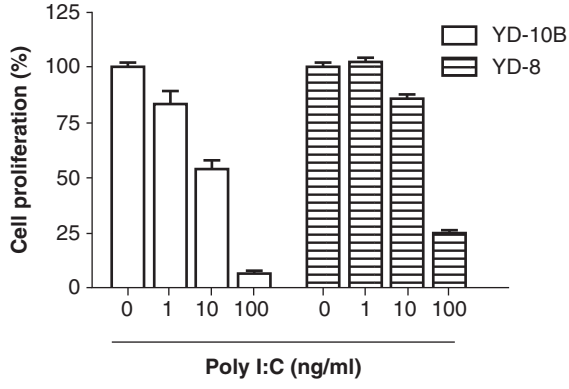

Figure 2. Cell proliferation assay. The cells were incubated with indicated doses of poly I:C for 24 h and cell proliferation was examined by MTT assay.

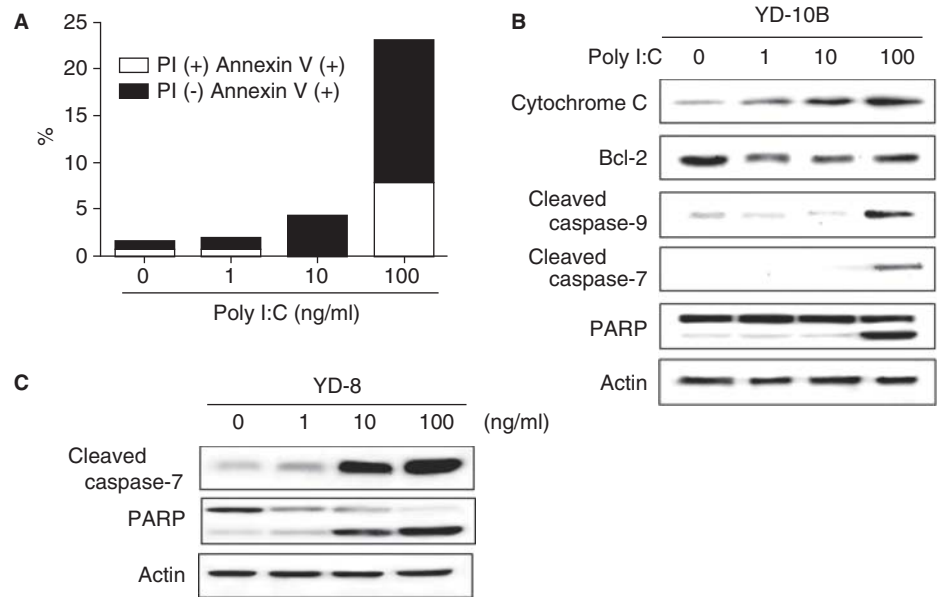

Figure 3. Flow cytometry and Western blot analysis. YD-10B cells were incubated with indicated doses of poly I:C for 24 h and stained with Alexa Fluor 488 Annexin V and PI for flow cytometry analysis. The ratio of PI(+)/Annexin V(+) or PI(−)/Annexin V(+) cells was obtained (A). And the protein levels of cytosolic cytochrome C, bcl-2, cleaved caspase-7/-9 and PARP were determined in YD-10B (B) and YD-8 (C) by Western blot.

treatment (Figure 1C). These findings indicated that TLR3 may be functionally expressed in OSCC cells.

Next, we examined the effect of TLR3 activation on the proliferation of OSCC cells. YD-10B and YD-8 cells were treated with various doses of poly I:C and cell proliferation was determined by MTT assay. As shown in Figure 2, poly I:C markedly reduced cell proliferation in a dose-dependent manner. Treatment of 100 ng/ml of poly I:C led to over 90% reduction in the proliferation of YD-10B cells and ~ 75% in YD-8 cells.

Previous studies demonstrated that poly I:C induced direct apoptosis in various cancer cells [6,7]. To know whether poly I:C induces apoptotic cell death in OSCC cells, YD-10B cells were treated with various doses of poly I:C for 24 h and flow cytometric analysis was performed. The ratio of both propidium iodide (PI) (−)/Annexin V(+) and PI(+)/Annexin V(+) cells was increased by poly I:C in a dose-dependent manner (Figure 3A), suggesting that TLR3 activation by poly I:C can induce apoptosis in OSCC cells.

Multiple pathways are involved in dsRNA-induced apoptosis. dsRNA-transfected pancreatic β-cells display PKR- and caspase-dependent apoptosis [18,19], whereas exogenous dsRNA-derived apoptosis in endothelial cells is mostly dependent on the extrinsic pathway [20]. The intrinsic (mitochondria-dependent) pathway of apoptosis is characterized by the release of cytochrome C from mitochondria to cytosol and

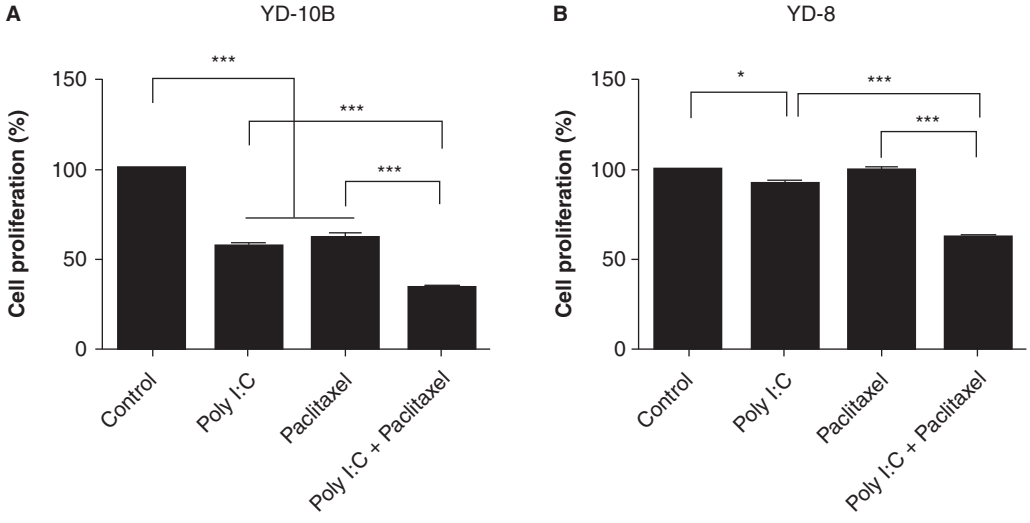

Figure 4. Cell proliferation assay. The cells were treated with poly I:C (10 ng/ml) alone, paclitaxel (0.25 μM) alone and combination of poly I:C and paclitaxel for 24 h and cell proliferation was examined by MTT assay. * $p < 0.05$, *** $p < 0.001$.

cleavage of caspase-9, whereas recruitment of death domains and activation of caspase-8 are involved in the extrinsic pathway. To clarify the precise mechanism of poly I:C-induced apoptosis in YD-10B cells, we performed Western blot analysis. Figure 3B showed that poly I:C increased release of cytochrome C to cytosol in a dose-dependent manner. An anti-apoptotic protein, bcl-2 expression, was decreased by poly I:C (Figure 3B). Cleavage of caspase-9, caspase-7 and PARP was also detected in YD-10B cells treated with 100 ng/ml poly I:C (Figure 3B). Also, poly I:C led to cleavage of caspase-7 and PARP in YD-8 cells (Figure 3C). These findings suggest that poly I:C may induce apoptosis in OSCC cells through a mitochondria-dependent pathway.

It has been demonstrated that poly I:C promotes drug-induced apoptosis via a TLR3-dependent pathway [16,21]. Therefore, we examined whether poly I:C enhances cell death of OSCC cells induced by an anti-cancer drug, paclitaxel. Results showed that combination treatment with poly I:C and paclitaxel more significantly induced cell death of YD-10B and YD-8 cells, compared to poly I:C or paclitaxel alone (Figure 4). These results suggest that the combination approach with poly I:C and anti-cancer drugs can maximize the anti-cancer effect and overcome the side-effects of the drugs by reducing the dose of the drugs in oral cancer patients.

In conclusion, we revealed here that human OSCC cell lines, YD-10B and YD-8, functionally expressed TLR3. Treatment of poly I:C significantly inhibited the proliferation of OSCC cells due to apoptosis via a mitochondria-dependent pathway. Furthermore, combination treatment of poly I:C and paclitaxel synergistically inhibited cell proliferation. Our results provide evidences that poly I:C can effectively inhibit cell growth by inducing apoptosis and its combination use with anti-cancer drugs can improve anti-cancer effect.

Acknowledgment

This study was supported by grants of the Korea Science and Engineering Foundation (KOSEF) funded by the Korea government (MOST) (Grant No. R13-2008-010-00000-0).

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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