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Growth inhibition by metformin in YD-38 oral cancer cells derived from Korean

한국인 유래 구강암 세포주 YD-38에서
metformin에 의한 세포성장 억제

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조선대학교 대학원

치 의 학 과

서 동 국

Growth inhibition by metformin in YD-38 oral cancer cells derived from Korean

지도교수 김 수 관

이 논문을 치의학 박사학위신청 논문으로 제출함.

2016년 4월

조선대학교 대학원

치 의 학 과

서 동 국

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2016년 6월

조선대학교 대학원

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ABSTRACT

Growth inhibition by metformin in YD-38 oral cancer cells derived from Korean

Seo, Dong Kuk

Advisor: Prof. Kim, Su-Gwan, D.D.S. Ph.D.

Department of Dentistry,

Graduate School of Chosun University

Metformin (1,1-dimethylbiguanide hydrochloride), derived from French lilac (*Galega officinalis*), is a first-line drug prescribed for patients with type 2 diabetes. It has been reported to have anti-cancer effects in the variety of cancer cells. However, metformin effects on oral cancer cells are not clearly established. The main goal of this study was to investigate the effect of metformin on cell growth and apoptosis induction in oral cancer cells derived from Korean.

To determine the effect of metformin on cell growth and apoptosis induction in oral cancer cells, it was examined by inhibition of cell growth (MTT assay), DNA fragmentation analysis and immunoblotting in YD-38 human oral cancer cells

derived from Korean. The results are as follows.

1. Treatment of metformin induced the inhibition of cell growth depended on the metformin treatment time and concentration in YD-38 cells.
2. Treatment of metformin induced the nuclear fragmentation in YD-38 cells.
3. Metformin promoted proteolytic cleavages of procaspase-3 with the increases in the amount of cleaved caspase-3.
4. Cleaved PARP was increased by metformin in YD-38 cells.
5. Treatment of YD-38 cells with metformin increased the level of Bax but decreased the level of Bcl-2.

These results suggest that the metformin can induce the suppression of cell growth and cell apoptosis in YD-38 human oral cancer cells derived from Korean through both the death receptor-mediated extrinsic apoptotic pathway and the mitochondria-mediated intrinsic apoptotic pathway, and that it may have potential properties for anti-cancer drug discovery.

KEY WORDS: Metformin, Cell death, Apoptosis, Anti-cancer therapy,

Oral cancer cells

I. INTRODUCTION

Metformin (1,1-dimethylbiguanide hydrochloride), derived from French lilac (*Galega officinalis*), is one of anti-diabetic drugs that belongs to the biguanide class, and a first-line drug prescribed for patients with type 2 diabetes [1-3]. It exerts its anti-diabetic effect by reducing hepatic glucose production [4,5]. Moreover, it is known to mimic the condition of nutrient starvation by blocking the electron transport chain complex I in mitochondria, and reducing the ATP/AMP ratio [6-8]. In addition, metformin is known to be used in the treatment of polycystic ovarian syndrome [9] or non-alcoholic fatty liver disease [10]. Recently, metformin has been reported to have anti-cancer effects in colon cancer, ovarian cancer, lung cancer, breast cancer and prostate cancer and so on [11-16]. Although it was shown to reduce the cancer cell viability, its action mechanisms remain to be determined. In addition, the effects of metformin on oral cancer are unclear.

Oral cancer is a major worldwide public health problem that may modify any part of the oral cavity, including the lips, tongue, mouth and throat [17-19]. Despite the introduction of novel therapeutic modalities to treat oral cancer, improvements in the long-term survival rates have been modest [20]. Therefore, multi-clinical studies, including surgical excision, radiation therapy and chemotherapy, have been designed and performed to help find the novel method for treating oral cancer [17,20,21]. And also, although the oral cancer research has made progress to date, the molecular mechanism underlying oral cancer is not understood yet.

The present study, therefore, examined the effects of metformin on cell growth

and the mechanism of cell death elicited by metformin in YD-38 human oral cancer cells derived from Korean.

II. MATERIALS AND METHODS

1. Materials

Metformin (1,1-dimethylbiguanide hydrochloride) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Anti-cleaved caspase-3, anti-poly(ADP-ribose) polymerase (PARP), anti-Bax, anti-Bcl-2 and anti- β -actin antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). All other analytical reagents purchased were of analytical grade.

2. Cell line and cell cultures

The human oral cancer cell line derived from Korean, YD-38, was provided by the Korean Cell Line Bank (Seoul, Korea) and cultured as according to the cell culture instructions provided by the Korean Cell Line Bank. Briefly, the YD-38 human oral cancer cells were grown in RPMI 1640 containing 10% fetal bovine serum (FBS). The cells were maintained as monolayers in plastic culture plates at 37° C in a humidified atmosphere containing 5% CO₂ [17].

3. Cell viability assay (MTT assay)

The cell viability assay (MTT assay) was applied to estimate the effect of metformin on YD-38 oral cancer cell proliferation and performed according to the

previously described method with minor modifications [22,23]. The cells were plated at a density of 5×10^3 cells/well in 24-well plates and allowed to attach to the well overnight. After overnight growth, the cells were treated with metformin at various concentrations and incubation times. Then, the cells were incubated for a further 4 hours in 20 μ l of 5 mg/ml MTT solution. To dissolve the formazan crystals transformed from MTT, the cells were resuspended in 150 μ l dimethyl sulfoxide (DMSO) and the optical density (OD) of the solution was determined using a spectrometer at an incident wavelength of 570 nm. Three separate experiments were performed for each concentration/exposure time combination.

4. DNA fragmentation analysis

Following treatment with 0 or 3 mM metformin for 72 hours, approximately 5×10^6 cells were collected and transferred to lysis buffer containing 100 mM NaCl, 10 mM EDTA, 300 mM Tris-HCl, pH 7.5, 200 mM sucrose, 0.5% SDS and 0.5 mg/ml proteinase K and incubated at 65°C. DNA was extracted with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1, v/v) and precipitated with ethanol. The DNA was resuspended in Tris-EDTA buffer, pH 8.0 containing 5 μ g/ml DNase-free RNase and incubated at 37°C for 1 hour. The DNA was visualized on 2% agarose gel in the presence of 0.5 μ g/ml ethidium bromide.

5. Immunoblotting

Immunoblotting was performed according to the previously described method

with minor modifications [23,24]. Briefly, the YD-38 cells were plated at a density of 5×10^3 cells/well in 24-well plates and allowed to attach to the well overnight. After overnight growth, the cells were treated with 0 or 3 mM metformin for 72 hours. After incubation, the cells were washed twice with ice-cold PBS and lysed using a RIPA buffer for protein extraction according to the manufacturer's instructions. The total protein concentrations were determined using the Bradford Assay (BioRad, Hercules, CA, USA). An equal amount of protein was resolved by 10% SDS-PAGE and transferred to PVDF membrane for immunoblotting. Membranes were blocked for 2 hours in 5% non-fat dry milk in TBST. The anti-cleaved caspase-3 (1:1000 dilution), anti-poly(ADP-ribose) polymerase (PARP) (1:1000 dilution), anti-Bax (1:1000 dilution), anti-Bcl-2 (1:1000 dilution) and anti- β -actin (1:5000 dilution) antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA) were used as the primary antibody. The immunoactivity was visualized using an Enhanced Chemi Luminescent System (ECL System, Amersham Biosciences, Piscataway, NJ, USA) and a Single Visual Enhancer System (Pierce, Rockford, IL, USA) to magnify the signal.

6. Data analysis

All experiments were performed at least three times. The results were presented as the mean \pm SEM. The statistical significance was analyzed by using Student's t-test for two groups and one way analysis of variance for multi-group comparisons. All statistical analyses were performed using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). Values of $p < 0.05$ were considered significant.

2. DNA fragmentation by metformin in YD-38 cells

Increased cellular apoptosis is only one among several possible mechanisms involved in reduced cell proliferation [25-28]. To determine if apoptosis is indeed the underlying mechanism for the reduced cell proliferation observed, the YD-38 cells treated with metformin were subjected to DNA fragmentation. As shown in Fig. 2, the formation of DNA ladder in the YD-38 cells treated with 3 mM metformin was observed, suggesting that metformin induces apoptotic cell death of YD-38 cells.

3. Activation of caspase-3 by metformin

The level of cleaved caspase-3 was examined by immunoblotting as caspase-3 is effector caspase of apoptotic cell death [28-31]. Treatment with 3 mM metformin for 72 hours significantly promoted the proteolytic cleavages of procaspase-3 in the YD-38 cells, with the increases in the amount of cleaved caspase-3 (Fig. 3).

4. Apoptosis mediated via PARP by metformin

To determine how metformin induce the extrinsic apoptosis of YD-38 cells, immunoblotting was performed to measure the expression of the PARP at the protein level. As shown in Fig. 4, cleaved-PARP was increased by metformin 3 mM compared to the control.

5. Apoptosis-related signal pathways by metformin

The levels of proteins that are highly relevant to understanding the apoptotic signaling pathways in YD-38 cells by metformin was measured by immunoblot analysis. The treatment of YD-38 cells with metformin increased the level of Bax protein expression (Fig. 5). On the other hand, the level of Bcl-2 protein expression in YD-38 cells stimulated with 3 mM metformin for 72 hours decreased (Fig. 6).

IV. DISCUSSION

Metformin, derived from French lilac, is an oral hypoglycemic drug that is widely used in the world [1-3]. Moreover, it is known to have anti-cancer effects [11-16]. In addition, type 2 diabetes treated with metformin showed a lower cancer-related mortality and a synergistic therapeutic effect for cancer when combined with chemotherapy [2,32-34]. Multiple mechanisms are reported to mediate the anti-cancer effects of metformin [35]. However, the metformin effects on oral cancer cells are not clearly established. In this study, therefore, the cytotoxic activity of metformin and the mechanism of cell death exhibited by metformin were examined in YD-38 human oral cancer cells derived from Korean. The present study demonstrated that the metformin can act as an apoptotic inducer in human oral cancer cells.

An MTT assay showed that the metformin inhibited the growth of YD-38 cells in a concentration- and a time-dependent manner (Fig. 1). This corresponded with the results of metformin that has anti-cancer effects via the suppression of cancer cell growth in many types of cancer cells [11-16]. These results speculated that metformin has cytotoxicity for oral cancer cells also and potential value for anti-cancer drug discovery.

Apoptosis, which is a major form of programmed cell death, plays an important role in regulating tissue development and homeostasis in eukaryotes [25-27], and the induction of apoptosis in cancer cells is an important indicator of the cancer treatment response [36]. Therefore, the researchers have screened many compounds

for their effects on apoptosis [36,37]. In the present study, treatment with 3 mM metformin induced the nuclear fragmentation in YD-38 cells (Fig. 2), suggesting apoptotic cell death. These results indicated that metformin inhibits the growth of YD-38 cells by activating cell apoptosis.

The activation of a family of intracellular cysteine proteases, known as caspases, is known to play an important role in the initiation and execution of apoptosis [29,30]. Among the caspases identified in mammalian cells, caspase-3 can serve as effector caspase of apoptotic cell death [29-31]. Caspase-3 is synthesized as an inactive proenzyme, which requires proteolytic activation to a cleaved enzyme of size 17 kDa [29-31]. The results of the present study show that low level of cleaved capase-3 was present in metformin-untreated YD-38 cells, and the amount of cleaved enzyme was increased after metformin treatment in YD-38 cells (Fig. 3). These results suggest that metformin induces apoptotic cell death by the activation of caspase-3 in YD-38 cells.

Apoptosis can occur via a death receptor-mediated extrinsic apoptotic pathway or a mitochondria-mediated intrinsic apoptotic pathway by treatment of anti-cancer agents [38-41]. Also, the cleaved PARP is an important regulatory factor of death receptor-mediated extrinsic apoptotic pathway [36]. In the present study, the expression of the cleaved PARP was up-regulated significantly by metformin in YD-38 cells (Fig. 4). Therefore, these results suggest that metformin-induced apoptosis in YD-38 cells is mediated by the death receptor-mediated extrinsic apoptotic pathway via the PARP.

Next, we assessed the effects of metformin on the expression of Bax and Bcl-2 in YD-38 cells. The Bax, pro-apoptotic proteins, and the Bcl-2,

anti-apoptotic mitochondrial protein, are important regulators of cytochrome *c* release from the mitochondria [36,42,43]. The Bcl-2 family is localized to the mitochondrial membrane and modulates apoptosis by permeabilizing the mitochondrial membrane, leading to the release of cytochrome *c* [44]. In the present study, treatment of YD-38 cells with metformin increased the level of Bax (Fig. 5) but decreased the level of Bcl-2 (Fig. 6). The Bax/Bcl-2 ratio is one of the indices of the mitochondria-mediated intrinsic apoptotic pathway [45]. The metformin-induced apoptosis appears to involve Bax/Bcl-2 signal transduction since metformin increased this ratio in YD-38 cells. Therefore, metformin is suggested to induce apoptosis in YD-38 cells involving the mitochondrial- and death receptor-signal transduction pathways. On the other hand, the mechanisms of apoptosis induced by metformin in YD-38 cells are not fully understood. Further studies are required to examine the precise cellular and molecular mechanisms of apoptosis induced by metformin.

In conclusion, these *in vitro* results suggest that the metformin inhibits cell proliferation and induces apoptotic cell death in YD-38 human oral cancer cells derived from Korean through both the death receptor-mediated extrinsic apoptotic pathway and the mitochondria-mediated intrinsic apoptotic pathway. Moreover, these results suggest that the metformin may provide a strategy for preventing and treating oral cancer and more research is needed to explore the molecular mechanisms.

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VI. FIGURE LEGENDS

Fig. 1. Concentration- and time-dependent effects of metformin on the cell viability in YD-38 human oral cancer cells. (A) Concentration-dependent effect of metformin on the cell viability in YD-38 cells. The YD-38 cells were treated with various concentrations of metformin or without metformin for 24 (circle), 48 (square) and 72 hours (triangle). (B) Time-dependent effect of metformin on the cell viability in YD-38 cells. The YD-38 cells were treated with 0.1 (circle), 0.3 (square), 1 (triangle), 3 (diamond) and 10 mM (hexagon) metformin for 0 - 72 hours. The cell viabilities were determined by the MTT assays. The percentage of cell viability was calculated as a ratio of $A_{570\text{nm}}$ of metformin treated cells and untreated control cells. Each data point represents the mean \pm SEM of three experiments. * $P < 0.05$ vs. control, ** $P < 0.01$ vs. control and *** $P < 0.001$ vs. control (the control cells measured in the absence of metformin).

Fig. 2. Fragmentation of internucleosomal DNA by metformin in YD-38 cells. The cells were treated with 0 or 3 mM metformin for 72 hours and nuclear DNA was subjected to agarose gel electrophoresis.

Fig. 3. Proteolytic cleavage of caspase-3 by metformin treatment in YD-38 cells. (A) Activity of cleaved caspase-3 by metformin was measured in YD-38 cells. The cells were treated with 0 or 3 mM metformin for 72 hours. The

cell lysate was prepared and analyzed by immunoblotting as described in "MATERIALS AND METHODS". (B) Quantitative data for (A) were analyzed by using Imagegauge 3.12 software after β -actin normalization.

Fig. 4. Activation of cleaved PARP by metformin treatment in YD-38 cells. (A) The activity of cleaved PARP by metformin was measured in YD-38 cells. The YD-38 cells were stimulated with 0 or 3 mM metformin for 72 hours, harvested and lyzed using a cell lysate buffer. (B) Quantitative data for (A) were analyzed by using Imagegauge 3.12 software after β -actin normalization.

Fig. 5. Regulation of Bax level by metformin treatment in YD-38 cells. (A) The activity of Bax by metformin was measured in YD-38 cells. (B) Quantitative data for (A) were analyzed by using Imagegauge 3.12 software after β -actin normalization.

Fig. 6. Regulation of Bcl-2 level by metformin treatment in YD-38 cells. (A) The activity of Bcl-2 by metformin was measured in YD-38 cells. (B) Quantitative data for (A) were analyzed by using Imagegauge 3.12 software after β -actin normalization.

VII. FIGURES

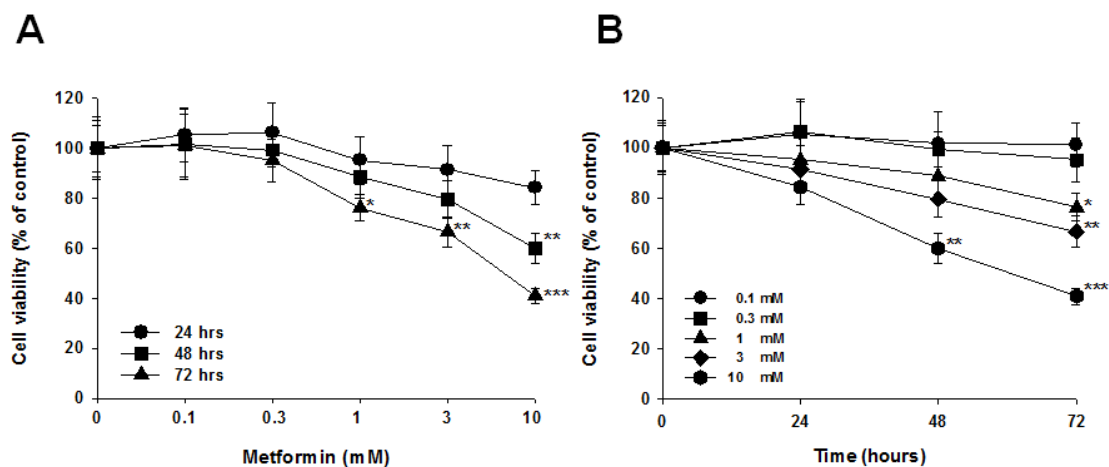


Fig. 1. Concentration- and time-dependent effects of metformin on the cell viability in YD-38 human oral cancer cells.

DNA fragmentation

Metformin (72 hours)

0 3 (mM)

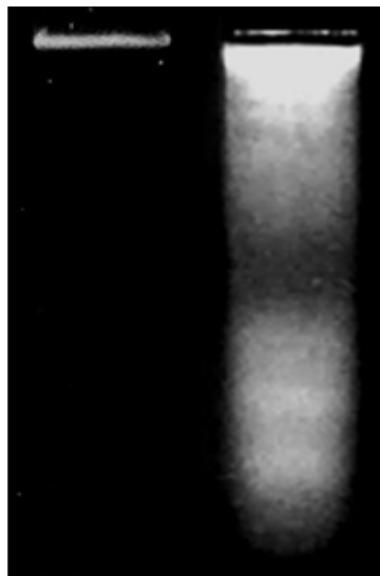


Fig. 2. Fragmentation of internucleosomal DNA by metformin in YD-38 cells.

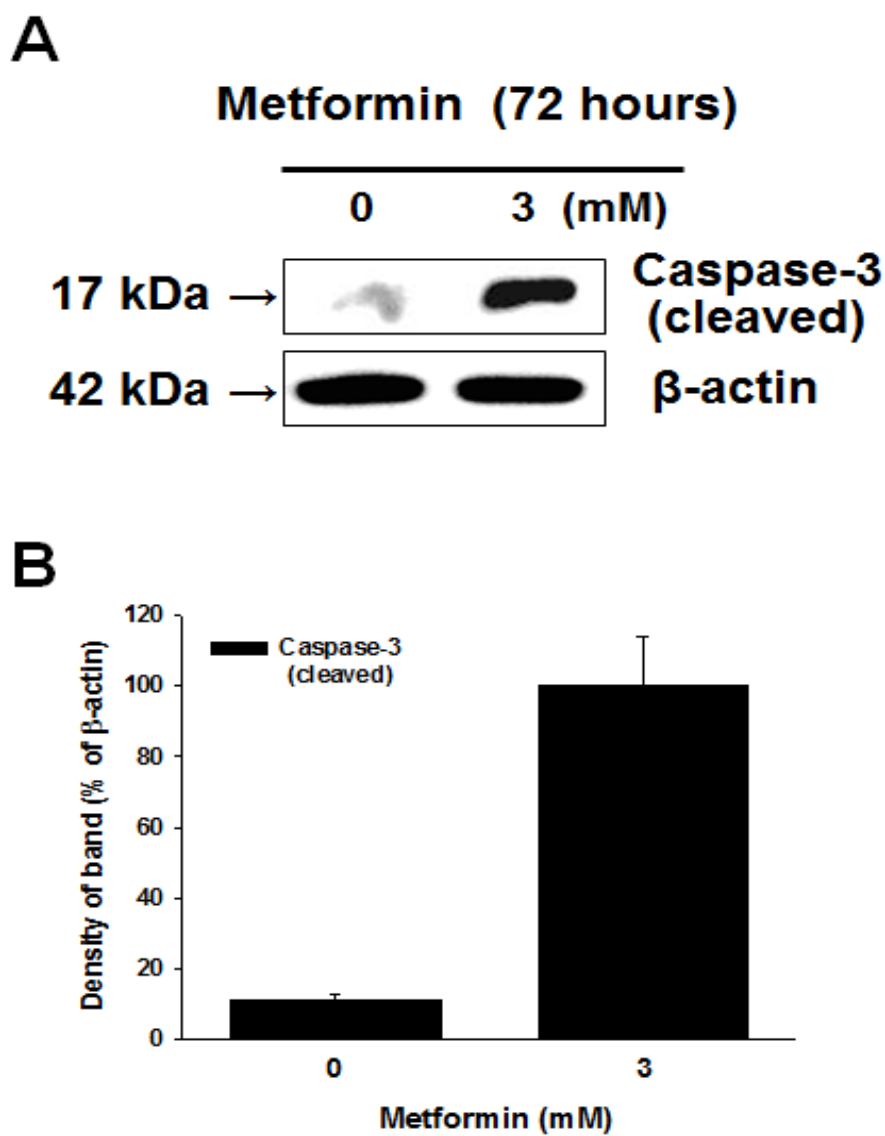


Fig. 3. Proteolytic cleavage of caspase-3 by metformin treatment in YD-38 cells.

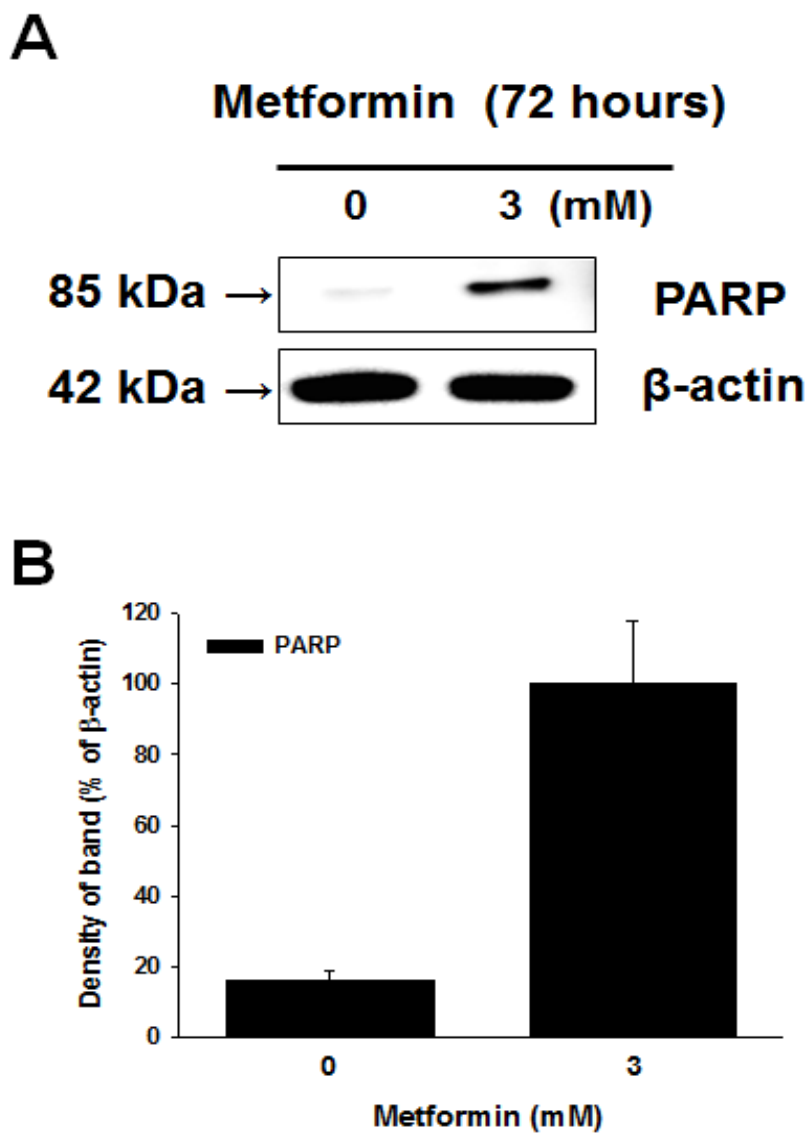
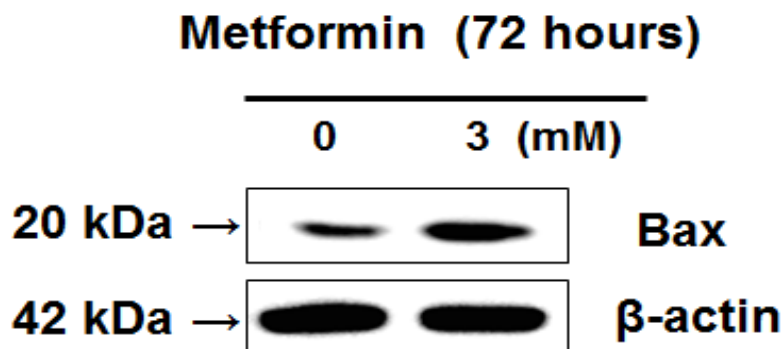


Fig. 4. Activation of cleaved PARP by metformin treatment in YD-38 cells.

A



B

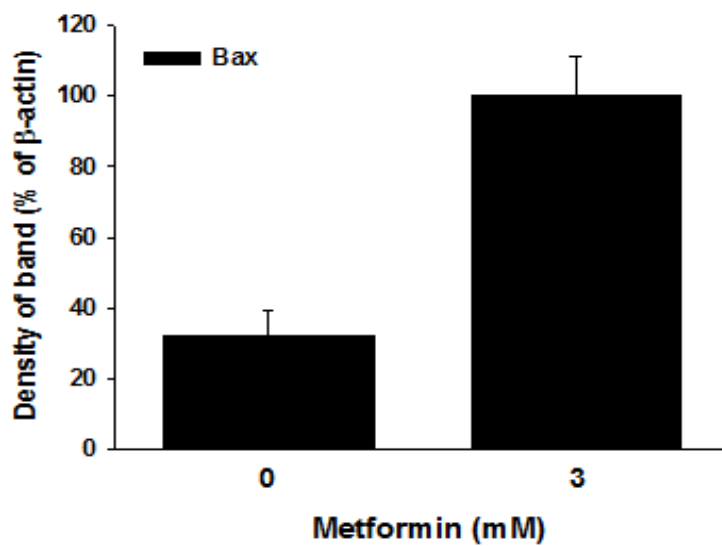
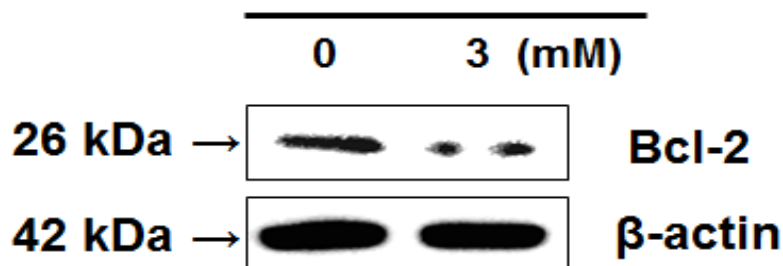


Fig. 5. Regulation of Bax level by metformin treatment in YD-38 cells.

A

Metformin (72 hours)



B

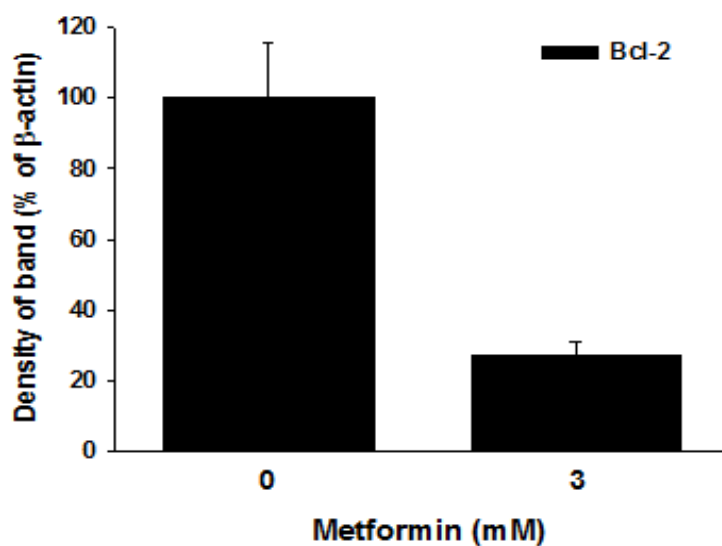


Fig. 6. Regulation of Bcl-2 level by metformin treatment in YD-38 cells.

ABSTRACT in KOREAN

한국인 유래 구강암 세포주 YD-38에서 metformin에 의한 세포성장 억제

서 동 국

조선대학교 대학원 치의학과

(지도교수 : 김 수 관)

French lilac(*Galega officinalis*)에서 유래한 metformin(1,1-dimethylbiguanide hydrochloride)은 type 2 당뇨병 치료제이며, 최근 들어 특정 암세포의 성장을 억제할 수 있다고 보고되었다. 그러나 구강암과 관련된 metformin에 관한 자료는 매우 부족하다. 따라서 본 연구에서는 한국인에서 유래한 구강암 세포주 YD-38 세포를 이용하여 metformin의 구강암세포 성장억제에 미치는 효과와 세포성장 억제기전을 분석하였다.

본 연구에서 metformin에 의한 구강암세포 성장억제와 그 기전을 조사하기 위해, YD-38 세포주에서 metformin을 이용하여 MTT 분석, DNA fragmentation 분석 및 immunoblotting 등을 시행하였다.

사람 구강암 세포주 YD-38에서 metformin은 구강암세포의 성장을 시간과 농도에 의존적으로 억제하였다. YD-38 세포에서 metformin은 핵의 파쇄 및 분절을 유도하였다.

YD-38 세포에 metformin을 처리한 실험군에서 procaspase-3 및 PARP의 proteolytic cleavage 현상을 확인할 수 있었다. YD-38 세포에 metformin을 처리한 실험군에서 Bax 단백질의 발현은 증가하였으나, Bcl-2 단백질의 발현은 감소하였다.

본 연구의 결과로 metformin은 한국인 유래 구강암 세포주 YD-38의 apoptosis를 유도하여 구강암세포 성장을 억제시키는 것으로 사료된다. 또한 본 연구의 결과로, metformin을 이용한 구강암세포의 성장억제에 관한 하나의 방향을 제시할 수 있을 것으로 사료된다.

중심어 : Metformin, 세포사, Apoptosis, 항암치료제, 구강암세포