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Hyperforin Inhibits Cell Growth by Inducing Intrinsic and Extrinsic Apoptotic Pathways in Hepatocellular Carcinoma Cells

I-TSANG CHIANG[1,2,3*], WEI-TING CHEN[4*], CHIH-WEI TSENG[5], YEN-CHUNG CHEN[2,6], YU-CHENG KUO[7], BI-JHIH CHEN[8], MAO-CHI WENG[9], HWAI-JENG LIN[10,11#] and WEI-SHU WANG[2,12,13#]

Departments of [1]Radiation Oncology, [6]Pathology, and [13]Medicine, and
[2]Cancer Medical Care Center, National Yang-Ming University Hospital, Yilan, Taiwan, R.O.C.;
[3]Department of Radiological Technology,
Central Taiwan University of Science and Technology, Taichung, Taiwan, R.O.C.;
[4]Department of Psychiatry, Zuoying Branch of Kaohsiung Armed Forces General Hospital,
Kaohsiung, Taiwan, R.O.C.;
[5]Division of Gastroenterology, Department of Internal Medicine, Dalin Tzu Chi Hospital,
Buddhist Tzu Chi Medical Foundation, Chia-Yi, Taiwan, R.O.C.;
[7]Radiation Oncology, Show Chwan Memorial Hospital, Changhua, Taiwan, R.O.C.;
[8]Department of Laboratory Medicine, Changhua Christian Hospital,
Changhua Christian Medical Foundation, Changhua, Taiwan, R.O.C.;
[9]Institute of Nuclear Energy Research, Atomic Energy Council, Taoyuan, Taiwan, R.O.C.;
[10]Department of Internal Medicine, Division of Gastroenterology and Hepatology, College of Medicine,
School of Medicine, Taipei Medical University, Taipei, Taiwan, R.O.C.;
[11]Department of Internal Medicine, Division of Gastroenterology and Hepatology,
Shuang-Ho Hospital, New Taipei, Taiwan, R.O.C.;
[12]National Yang-Ming University School of Medicine, Taipei, Taiwan, R.O.C.

Abstract. *The aim of the present study was to investigate the antitumor effect and mechanism of action of hyperforin in hepatocellular carcinoma (HCC) SK-Hep1 cells in vitro. Cells were treated with different concentrations of hyperforin for different periods of time. Effects of hyperforin on cell viability, apoptosis signaling, and expression of anti-apoptotic and proliferative proteins [cellular FLICE-like inhibitory protein (c-FLIP), X-linked inhibitor of apoptosis protein (XIAP), myeloid cell leukemia 1(MCL1), and cyclin-D1] were investigated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, flow cytometry, and western blotting. Hyperforin significantly inhibited cell viability and expression of anti-apoptotic and proliferative proteins. We also found that hyperforin significantly induced accumulation of cells in sub-G_1 phase, loss of mitochondrial membrane potential, and increased levels of active caspase-3, and caspase-8. Taken together, our findings indicate that hyperforin triggers inhibition of tumor cell growth by inducing intrinsic and extrinsic apoptotic pathways in HCC SK-Hep1 cells.*

Apoptosis, or programmed cell death, can be divided into initiation, effector, and degradation phases (1). The process of apoptosis is activated by a variety of physiologic and non-physiological stimuli through extrinsic and intrinsic pathways. Morphological characteristics of apoptosis include cell shrinkage, surface blebbing, chromatin condensation, chromosomal DNA fragmentation, and formation of apoptotic bodies (2). Anticancer agents kill cancer cells by inducing extrinsic and intrinsic apoptotic pathways. Tumoricidal effects

[*#]These Authors contributed equally to this study.

Correspondence to: Professor Wei-Shu Wang, M.D., Ph.D., Department of Medicine, National Yang-Ming University Hospital, No.152, Xinmin Rd., Yilan City, Yilan County 260, Taiwan, R.O.C. Tel: +886 39325192 ext. 1621, Fax: +886 39351838, e-mail: 11313@ymuh.ym.edu.tw and Hwai-Jeng Lin, M.D., Department of Internal Medicine, Division of Gastroenterology and Hepatology, Shuang-Ho Hospital, No.901, No. 291 Zhongzheng Rd. Zhonghe District, New Taipei City, 23561, Taiwan, R.O.C. Tel: +886 52648000 ext: 5333, Fax: +886 52838370, e-mail: buddhistlearning@gmail.com

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of anticancer agents can be inhibited by anti-apoptotic proteins in cancer cells and these result in treatment failure (3). Hepatocellular carcinoma (HCC) is among the most common lethal types of cancer worldwide and resistant to conventional chemotherapy through high expression of anti-apoptotic proteins such as cellular FLICE-like inhibitory protein (c-FLIP), myeloid cell leukemia 1 (MCL1), and X-linked inhibitor of apoptosis protein (XIAP). Therefore, development of agents facilitating induction of apoptosis may be beneficial for patients with HCC (4-7).

Antidepressants can improve mood disorders, such as major depression, anxious disorder, and dysthymia by altering the reuptake of neurotransmitters at the synapse. Several recent studies indicated that antidepressants not only alleviate mood disorders but also influence tumor progression (8, 9). Frick *et al*. suggested that antidepressants are able to modulate apoptotic mechanism and activate antitumor immunity to inhibit tumor growth (8). Fluoxetine, a selective serotonin reuptake inhibitor, has been found to suppress tumor growth by inducing apoptosis of HCC and glioblastoma cells (10, 11). Fang *et al*. suggested that mirtazapine, a noradrenergic and specific serotonergic antidepressant, increases expression of interlukin-12 (IL12) and interferon-gamma (INFγ) in addition to enhancing $CD4^+$ and $CD8^+$ T-cell infiltration within cancer tissue, and inhibits tumor growth in colorectal cancer *in vivo* (12).

Hyperforin, a polyprenylated acylphloroglucinol isolated from St. John's wort, has antidepressant activity by blocking the reuptake of serotonin, dopamine, noradrenaline, γ-aminobutyric acid, and glutamate. In addition, hyperforin has antitumor effect in various types of cancer cells (13). Donà *et al*. found that hyperforin inhibits expression of metastasis-associated proteins, such as urokinase-type plasminogen activator , and matrix metalloproteinases (MMPs) 2 and 9 while reducing lung metastasis (14). Hyperforin also acts as an apoptotic inducer to promote caspase-mediated apoptosis in breast cancer and myeloid tumor cells (15, 16). However, whether hyperforin can inhibit tumor cell growth by inducing apoptosis in HCC is not known. The aim of the present study was to investigate the antitumor effect and mechanism of action of hyperforin in HCC SK-Hep1 cells by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), flow cytometry, and western blotting.

Materials and Methods

Chemicals and agents. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, and penicillin-streptomycin (PS) were bought from Gibco/Life Technologies (Carlsbad, CA, USA). Propidium iodide (PI), CaspGLOW™ Fluorescein Active Caspase-3 Staining Kit, and CaspGLOW™ Red Active Caspase-8 Staining Kit were bought from Biovision (Mountain View, CA, USA). 3,3'-Dihexyloxacarbocyanine Iodide ($DiOC_6$) was obtained from Enzo Life Sciences (Farmingdale, NY, USA). Hyperforin and MTT were purchased from Sigma-Aldrich (St. Louis, MO, USA). RNase was obtained from Fermentas (St. Leon-Rot, Baden-Württemberg, Germany). Primary antibodies to c-FLIP and cyclin D1 were bought from Cell Signaling Technology (Beverly, MA, USA). Primary antibody to XIAP was obtained from Thermo Fisher Scientific (Fremont, CA, USA). Primary antibody to β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibody to MCL1 was bought from BioVision (Milpitas, CA, USA). Secondary antibodies were bought from Jackson ImmunoResearch (West Grove, PA, USA).

Cell culture. SK-Hep1 cells were obtained from Professor Jing-Gung Chung at the Department of Biological Science and Technology, China Medical University, Taichung, Taiwan. Cells were cultured in DMEM containing 10% FBS, 2 mM L-glutamine, and PS (100 U/ml and 100 μg/ml) at 37˚C in a humidified atmosphere containing 5% CO_2 (17).

MTT assay. SK-Hep1 cells were plated into 96-well plates at 3×10^4 cells/well and incubated overnight. Cells were treated with different concentration of hyperforin [0-10 μM in 0.1% dimethyl sulfoxide (DMSO)] for 24 or 48 h. The effects of hyperforin on cell viability was evaluated with MTT assay, as described by Chen *et al*. (18).

Analysis of sub-G_1 population. SK-Hep1 were plated into 12-well plates at 2×10^5 cells/well and incubated overnight then treated with 5 μM hyperforin for different time intervals. Cells were harvested by centrifugation and fixed with 70% ethanol and incubated overnight at −20˚C. Cells were washed twice with phosphate-buffered saline (PBS) and then re-suspended in 500 μl of PI working solution (40 μg/ml PI, 100 μg/ml RNase, and 1% Triton X-100 in PBS) in the dark at room temperature for 1 h. Analysis of cell-cycle distribution was performed by using flow cytometry (FACS101, Becton Dickinson FACScan, Franklin Lakes, NJ, USA) as described by Ma *et al*. (19).

Detection of active caspase-3. SK-Hep1 were plated into 12-well plates at 2×10^5 cells/well and incubated overnight then treated with 5 μM hyperforin for different time intervals. Cells were collected by centrifugation and washed with PBS, and re-suspended in 300 μl of $Asp(OCH_3)$-$Glu(OCH_3)$-Val-$Asp(OCH_3)$-fluoromethyl ketone (DEVD-FMK), conjugated to fluorescein isothiocyanate (FITC) working solution (1 μl FITC-DEVD-FMK in 300 μl PBS) for 0.5 h at 37˚C in a humidified atmosphere containing 5% CO_2. Cells were then harvested by centrifugation and washed twice with wash buffer and then re-suspended in 300 μl of wash buffer. Detection of active caspase-3 was performed by using flow cytometry (FACS101, Becton Dickinson FACScan; Becton Dickinson, Franklin Lakes, NJ, USA) with FL-1 channel (20).

Detection of mitochondrial membrane potential (MMP). SK-Hep1 were plated into 12-well plates at 2×10^5 cells/well and incubated overnight then treated with 5 μM hyperforin for different time intervals. Cells were collected by centrifugation and washed with PBS, and resuspended in $DiOC_6$ working solution (4 μM $DiOC_6$ in 500 μl PBS) in the dark at 37˚C for 0.5 h. Analysis of MMP was performed using flow cytometry (FACS101, Becton Dickinson FACScan) as described by Hsu *et al*. (21).

Detection of active caspase-8. SK-Hep1 were plated into 12-well plates at 2×10^5 cells/well and incubated overnight then treated with

5 μM hyperforin for different time intervals. Cells were collected by centrifugation and washed with PBS, and re-suspended in 301 μl of IETD-FMK conjugated to sulfo-rhodamine (Red-IETD-FMK) working solution (1 μl Red-IETD-FMK in 300 μl PBS) for 0.5 h at 37˚C in a humidified atmosphere containing 5% CO_2. Cells were harvested by centrifugation and washed twice with wash buffer and then re-suspended in 300 μl of wash buffer. Detection of active caspase-8 was performed by using flow cytometry (FACS101, Becton Dickinson FACScan) with FL-2 channel (22).

Western blotting assay. SK-Hep1 cells (3×10^6) were plated into 10 cm diameter dishes and incubated overnight then treated with 5 μM hyperforin for different time intervals. Lysis buffer (50 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5% NP-40, and 1 mM phenylmethanesulfonyl fluoride) was used to extracted total protein from cells in each group. Protein levels of c-FLIP, XIAP, MCL1, and cyclin D1 were determined with western blotting assay as described by Lai *et al*. (23). ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used for quantification of protein bands.

Statistical analysis. Difference of means between experimental and control groups was tested using Student's *t*-test and considered significant with *p*-value of 0.05 or less. Data are presented as the mean±standard error.

Results

Hyperforin induces cytotoxicity in SK-Hep1 cells. Hyperforin significantly reduced cell viability in SK-Hep1 cells in a time-dependent manner. Figure 1 shows that hyperforin treatment (2.5-10 μM) significantly reduced cell viability by 29-55% and 54-74% at 24 h and 48 h as compared to controls, respectively.

Hyperforin induces apoptosis of SK-Hep1 cells. Accumulation of cells at sub-G1 phase and activation of casapse-3 and are correlated with apoptosis (24, 25). In order to verify whether hyperforin induces apoptosis of SK-Hep1 cells, detection of active caspase-3 and the sub-G_1 cell population were performed by using flow cytometry. Figure 2 indicates hyperforin significantly increased the sub-G_1 cell population and level of active caspase-3 in a time-dependent manner as compared to the control.

Hyperforin induces apoptosis through intrinsic and extrinsic pathways in SK-Hep1 cells. Loss of MMP is a characteristic of the intrinsic apoptotic pathway while activated caspase-8 is a specific marker of the extrinsic apoptotic pathway (26, 27). Detection of MMP and active caspase-8 was used to evaluate the mechanism of hyperforin-induced apoptosis of SK-Hep1 cells. Figure 3A shows that hyperforin significantly enhanced loss of MMP as compared to controls at 24 h and 48 h. Figure 3B indicates that hyperforin significantly induced activation of caspase-8 in a time-dependent manner as compared to controls.

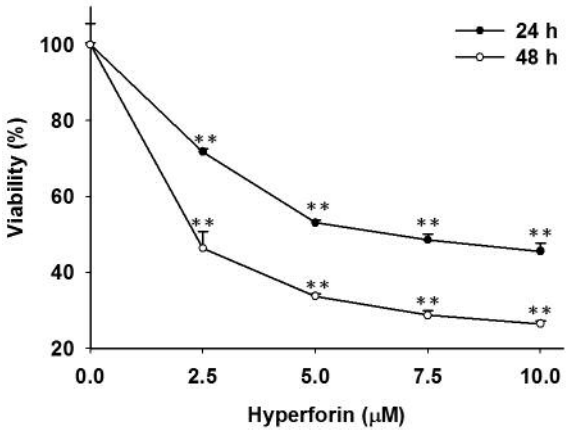

Figure 1. *The effects of hyperforin on viability of SK-Hep1 cells. Cells were treated with different concentrations (in 0.1% dimethyl sulfoxide) of hyperforin for 24 and 48 h. Cell viability was evaluated with the MTT assay. **Significantly different at p<0.01 compared to controls.*

Hyperforin inhibits expression of anti-apoptotic and proliferative proteins in SK-Hep1 cell. Western blotting assay was used to evaluate whether hyperforin-induced apoptosis is associated with inhibition of anti-apoptotic protein expression. Cyclin D1, a proliferative protein, acts as active switch to initiate cell-cycle progression (28). Therefore, we also investigated the effect of hyperforin on expression of cyclin D1 in addition to anti-apoptotic proteins. Figure 4 shows that hyperforin significantly reduced protein levels of c-FLIP, XIAP, MCL1, and cyclin D1 in a time-dependent manner as compared to controls.

Discussion

Hyperforin, a bioactive compound with antidepressive effect, has been indicated to inhibit tumor growth in breast cancer and myeloid tumor by induction of caspase-mediated apoptosis (15, 16). However, whether hyperforin can suppress tumor cell growth through induction of apoptosis in HCC is ambiguous. Therefore, we investigated the antitumor effect and mechanism of hyperforin in HCC SK-Hep1 cells. To begin with, we found that hyperforin induces cytotoxicity in SK-Hep1 cells (Figure 1). Hyperforin triggers accumulation of cells at sub-G_1 phase and increases the level of active caspase-3 (Figure 2). Furthermore, hyperforin elicits loss of MMP and activation of caspase-8 (Figure 3). Finally, hyperforin reduces expression of anti-apoptotic and proliferative proteins (c-FLIP, XIAP, MCL1, and cyclin D1).

High expression of anti-apoptotic and proliferative proteins including c-FLIP, XIAP, MCL1, and cyclin D1 is observed in HCC and related to poor prognosis of patients (5-7). Fleischer *et al*. found increased MCL1 protein

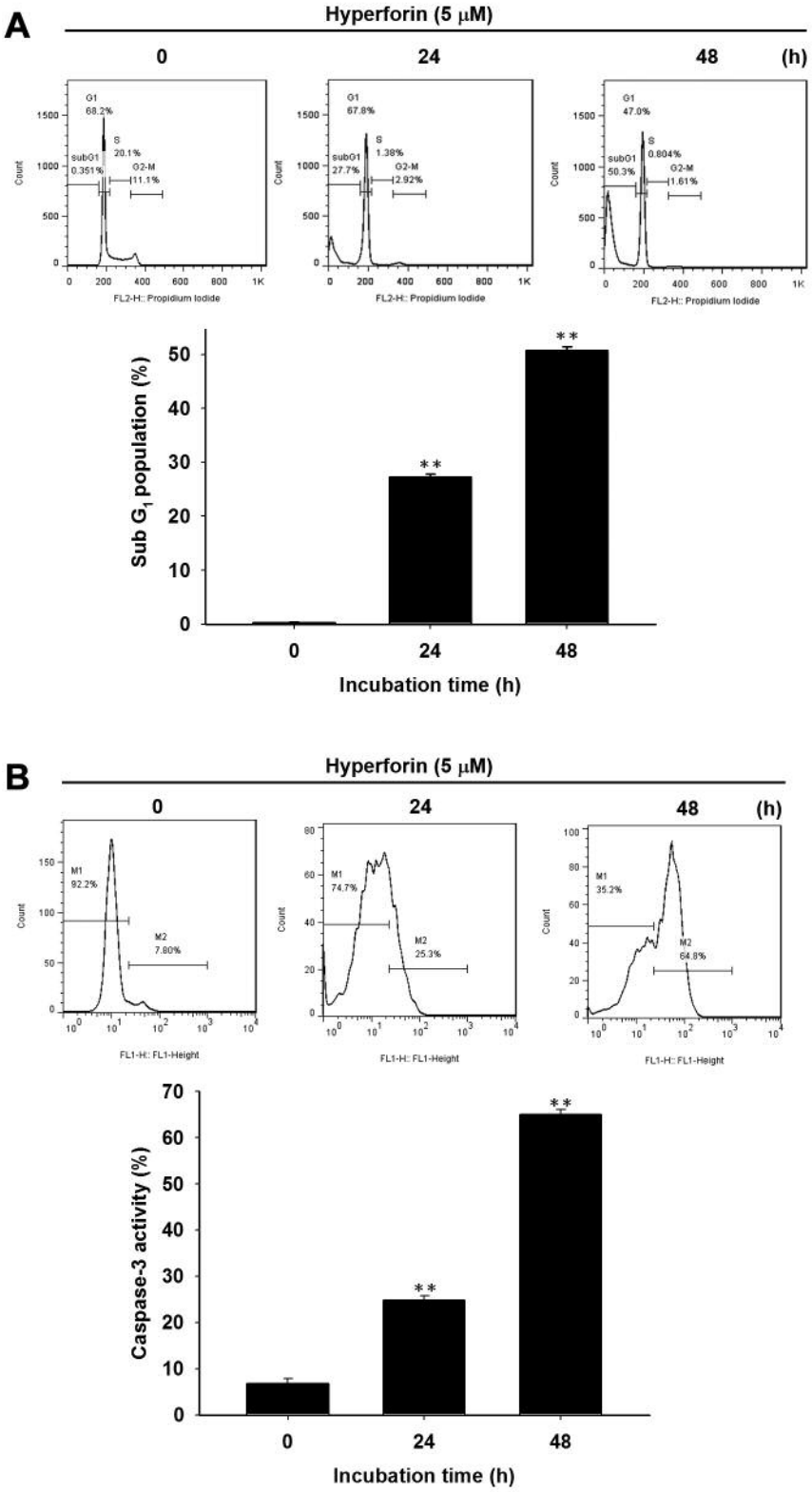

Figure 2. *The effects of hyperforin on the sub-G_1 population (A) and activation of caspase-3 (B) in SK-Hep1 cells. Cells were treated with 5 μM hyperforin for 24 and 48 h. Analysis of Sub-G_1 population and detection of active caspase-3 was performed by using flow cytometry. **Significantly different at p<0.01 compared to controls.*

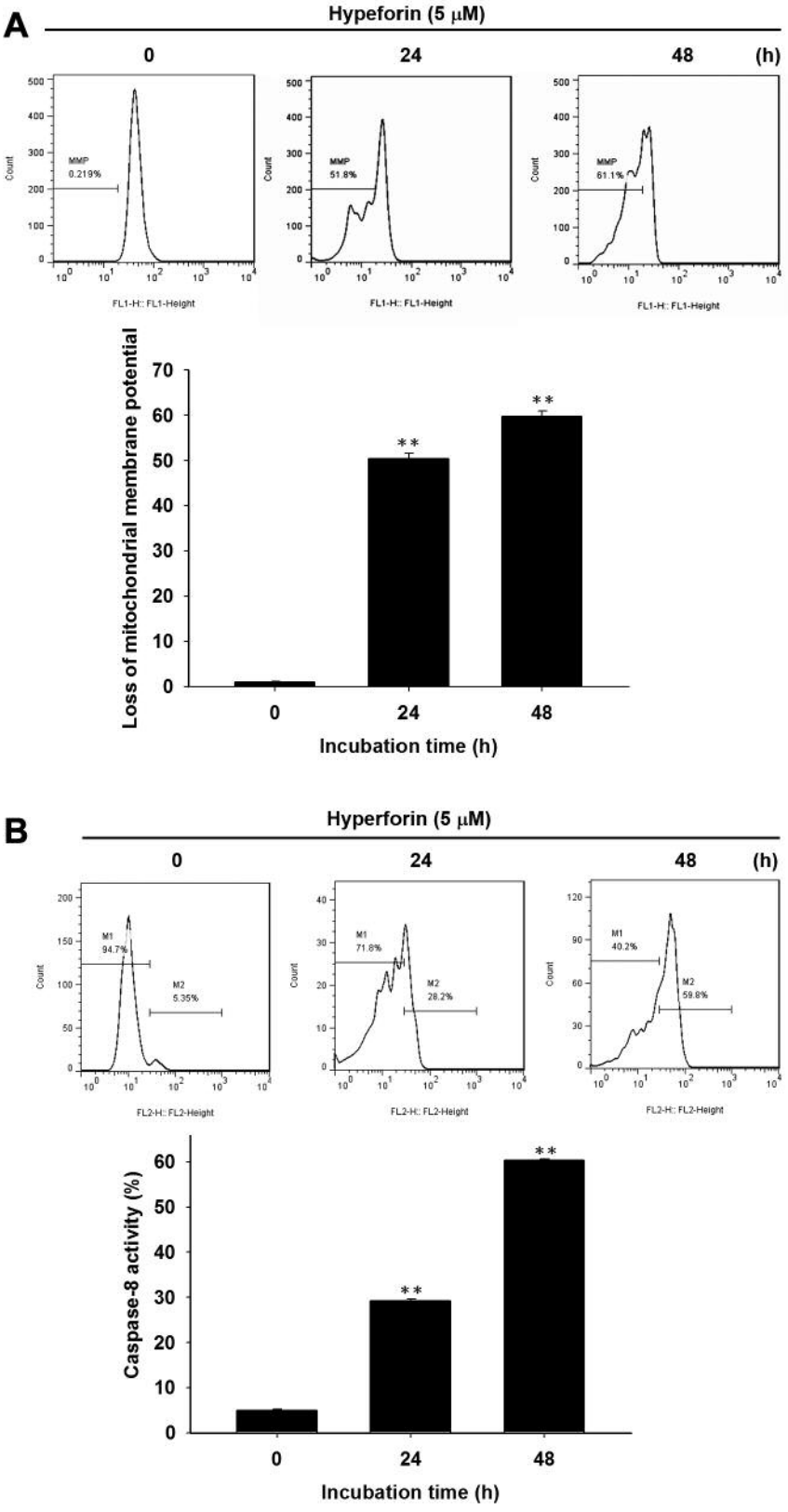

Figure 3. *The effects of hyperforin on mitochondrial membrane potential (A) and activation of caspase-8 (B) in SK-Hep1 cells as detected by using flow cytometry. **Significantly different at p<0.01 as compared to controls.*

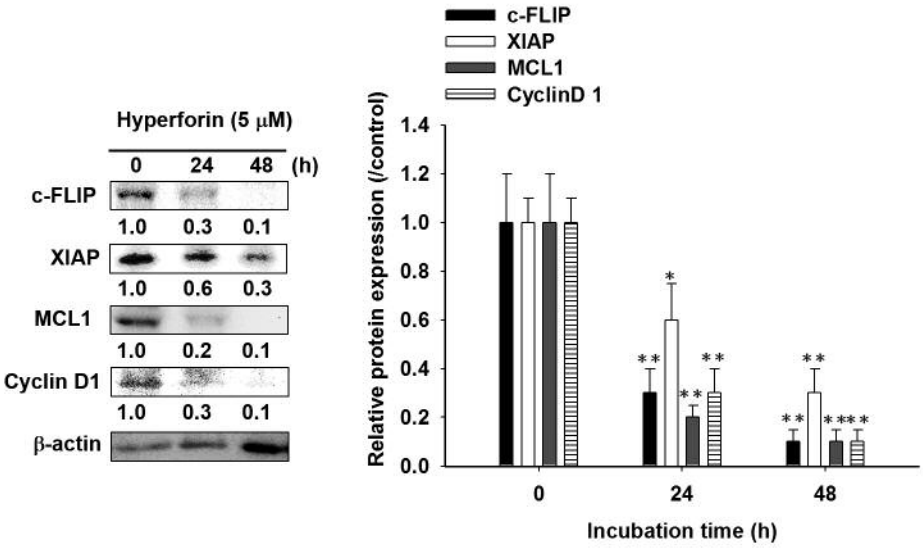

Figure 4. *The effects of hyperforin on expression of anti-apoptotic and proliferative proteins in SK-Hep1 cells. Changes of cellular FLICE-like inhibitory protein (c-FLIP), myeloid cell leukemia 1 (MCL1), and X-linked inhibitor of apoptosis protein (XIAP) and cyclin D1 protein. Protein levels were evaluated with western blotting assay. Significantly different at *p<0.05 and **p<0.01 as compared to controls.*

expression in human HCC tissue compared to adjacent non-tumor tissues and suggested that MCL1 is an anti-apoptotic factor for HCC (6). Che *et al*. found co-expression of XIAP and cyclin D1 proteins correlated with poor prognosis in patients with HCC (7). Du *et al*. found that overexpression of c-FLIP protein implied a lower probability of recurrence-free survival in patients with HCC and specific silencing of c-FLIP gene enhanced drug-induced apoptosis of HCC cells (5). Our study results demonstrated that hyperforin significantly reduces expression of c-FLIP, XIAP, MCL1, and cyclin D1 (Figure 4). Merhi *et al*. found that hyperforin inhibited expression of anti-apoptotic protein B-cell lymphoma 2 (BCL2) in a dose-dependent manner while levels of MCL1 in human myeloid tumor cells appeared to be reduced by hyperforin at a concentration of 2 μg/ml (16). Our data show that 5 μM hyperforin suppressed expression of MCL1 protein in a time-dependent manner (Figure 4).

The loss of MMP is required for induction of the intrinsic apoptotic pathway. Apoptotic protein cytochrome *c*, an activator of downstream caspases, is bound to the inner mitochondrial membrane by anionic phospholipid cardiolipin. Cytochrome *c* released from mitochondria is associated with loss of MMP and initiates intrinsic apoptosis (29). Active caspase-8, which modulates activation of caspase-3 and caspase-7, is essential for induction of extrinsic apoptosis by different death receptors (27). Schempp *et al*. found loss of MMP and cytochrome *c* release are enhanced but activation of caspase-8 is not affected by hyperforin (15). Therefore, they suggested that hyperforin induces apoptosis through intrinsic apoptotic pathway in breast carcinoma MT-450 cells. Our results indicated that hyperforin induces both loss of MMP and increase of active caspase-8 in SK-Hep1 cells (Figure 3). We suggest that hyperforin not only inhibits expression of anti-apoptotic and proliferative proteins (c-FLIP, XIAP, MCL1, and cyclin D1) but also induces intrinsic and extrinsic apoptosis of SK-Hep1 cells.

In conclusion, this study indicated that hyperforin, as an apoptotic inducer, can inhibit tumor cell growth through induction of intrinsic and extrinsic apoptotic pathways. We propose that hyperforin may be a potential anticancer agent for patients with HCC.

Acknowledgements

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