FEBS Letters 581 (2007) 865–871

Surfactin from *Bacillus subtilis* displays anti-proliferative effect via apoptosis induction, cell cycle arrest and survival signaling suppression

Seo-young Kim[a], Joo Young Kim[b], Seol-Hee Kim[c], Hyun Jin Bae[a], Hwaseon Yi[a], Sang Hong Yoon[d], Bon Sung Koo[d], Moosik Kwon[a], Jae Youl Cho[b], Choong-Eun Lee[c], Sungyoul Hong[a,*]

[a] *Department of Genetic Engineering, Sungkyunkwan University, Suwon 440-746, Republic of Korea*
[b] *School of Bioscience and Biotechnology, Kangwon National University, Chuncheon 200-701, Republic of Korea*
[c] *Department of Biological Science and Institute for Basic Science, Sungkyunkwan University, Suwon 440-746, Republic of Korea*
[d] *Microbial Function Team, National Institute of Agricultural Biotechnology, Suwon 441-707, Republic of Korea*

Received 6 November 2006; revised 16 January 2007; accepted 22 January 2007

Available online 2 February 2007

Edited by Varda Rotter

Abstract **The effect of surfactin on the proliferation of LoVo cells, a human colon carcinoma cell line, was examined. Surfactin strongly blocked the proliferation of LoVo cells by inducing pro-apoptotic activity and arresting the cell cycle, according to several lines of evidence on DNA fragmentation, Annexin V staining, and altered levels of poly (ADP-ribose) polymerase, caspase-3, $p21^{WAF1/Cip1}$, p53, CDK2 and cyclin E. The anti-proliferative activity of surfactin was mediated by inhibiting extracellular-related protein kinase and phosphoinositide 3-kinase/Akt activation, as assessed by phosphorylation levels. Therefore, our data suggest that surfactin may have anti-cancer properties as a result of its ability to downregulate the cell cycle and suppress its survival.**

Keywords: Surfactin; Anti-cancer effect; Apoptosis; Cell cycle arrest; Phosphoinositide 3-kinase; Akt

1. Introduction

A subtle balance between cell proliferation and death is absolutely necessary for keeping physiologically normal cell growth, whereas aberrant upregulation of cell proliferation is linked to tumorigenesis and cancerous states. For cell proliferation, cell cycle must progress properly. The activation of cyclin-dependent kinases (CDKs 4/6, 2, and 1) via binding to cyclins (D, E, A and B) and the phosphorylation of E2F and retinoblastoma are required for cell cycle progression [1,2]. CIP/KIP family members ($p21^{WAF1/Cip1}$, $p27^{KIP1}$ and $p57^{KIP2}$) are involved in negatively controlling the formation of the CDK4/6-cyclin D complex, reducing its kinase activity in p53-dependent or independent manners during the G_1/S phase of the cell cycle [3].

Apoptosis is a fundamental form of cell death that occurs without inducing inflammation. This event is readily distinguished by morphological and biochemical features, such as membrane blebbing and internucleosomal DNA cleavage [4,5]. Pro-apoptotic events are managed by two different pathways, extrinsic and intrinsic pathways [6]. The death receptor complex [Fas ligands and CD95 (APO-1/Fas)]-mediated cellular process linked to caspase-8 and caspase-3 is a typical extrinsic pathway. Intrinsic pathway is triggered by internal death signals managed by mitochondrial components such as cytochrome *c* together with Apaf-1. These two pathways come together at caspase-3 and this cleavage enzyme mediates various biochemical and morphological changes via cleaving intracellular substrates such as poly (ADP-ribose) polymerase (PARP), a sensitive marker of caspase-mediated apoptosis [7].

Intracellular signals regulating cell survival and death are now largely understood, owing to numerous studies. Of the signaling molecules, phosphoinositide 3-kinase (PI3K), Akt, mitogen-activated protein kinases (MAPK) [such as extracellular-related protein kinase (ERK), p38 and C-Jun N-terminal kinase (JNK)] and IKK (IκB kinase)/IκB/nuclear factor (NF)-κB are regarded as important components in regulating the expression level and activation of apoptosis- and cell cycle-regulating proteins, including $p21^{WAF1/Cip1}$, Bcl-2, p53, cyclin E and CDK [8,9].

Surfactin (M.W.: 1036 Da) is a cyclic lipopeptide biosurfactant containing heptapeptide (LLDLLDL) and a beta-hydroxy fatty acid. This biosurfactant has been reported to possess various biological activities, such as anti-mycoplasma, anti-viral, anti-fibrinogenic, anti-hypercholesterolemic and anti-inflammatory functions [10–13]. Although surfactin is considered to be a potential antitumor agent [14], its particular effects on cancer cells have not yet been proven. How this molecule can be effective in various biological events is still largely unknown, however, it is speculated that the structural and lipophilic properties of surfactin may affect the stability of biological membranes.

In the previous study, we identified several bioactive metabolites displaying anti-inflammatory, anti-cancer and anti-angiogenesis (unpublished data) activities from Korean traditional shrimp-fermented foods. One of these metabolites was found to be surfactin produced from a bacterium, *Bacillus subtilis* strain JKK238. In the present study, therefore, we aimed to explore the anti-proliferative activity (cell cycle arrest and

*Corresponding author. Fax: +82 31 290 7895.
E-mail address: syhong@skku.ac.kr (S. Hong).

Abbreviations: CDKs, cyclin-dependent kinases; ERK, extracellular-related protein kinase; IKK, IκB kinase; JNK, C-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PARP, poly (ADP-ribose) polymerase; PI3K, phosphoinositide 3-kinase

0014-5793/$32.00
doi:10.1016/j.febslet.2007.01.059

induction of apoptosis) of surfactin against human colon cancer (LoVo) cells and its anti-cancer mechanism at molecular levels in detail. This study is the first report of the anti-cancer activity of bacterium-derived biosurfactant assessed at the molecular level.

2. Materials and methods

2.1. Materials

Surfactin and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were purchased from Sigma Chemical Co. (St. Louis, MO). SP600125 (a JNK inhibitor), SB203580 (a p38 inhibitor), U0126 (an ERK inhibitor), wortmannin (a PI3K inhibitor) and LY294002 (a PI3K inhibitor) were obtained from Calbiochem. (San Diego, CA). Antibodies to ERK, p38, JNK, Akt, cleaved caspase-3 (Asp175), cleaved PARP (Asp214) and β-actin, and phospho-specific antibodies to ERK, p38, JNK, IκBα, Akt and p85 were purchased from Cell Signaling Technology (Beverly, MA). Antibody to PARP was from BD Pharmingen (San Diego, CA). LoVo cells, the human colon cancer cell line, cultured in RPMI 1640 supplemented with 10% FBS and antibiotics [penicillin (100 U/ml)/streptomycin (100 μg/ml)] without mycoplasma contamination, were purchased from ATCC (Manassas, VA).

2.2. Cytotoxicity assay

See Supplementary Materials.

2.3. Reverse transcription polymerase chain reaction (RT-PCR)

See Supplementary Materials.

2.4. Phase-contrast microscopy and DAPI staining

See Supplementary Materials.

2.5. Annexin V staining

See Supplementary Materials.

2.6. DNA fragmentation assay

See Supplementary Materials.

2.7. Immunoblotting

See Supplementary Materials.

2.8. Statistical analysis

See Supplementary Materials.

3. Results

3.1. Surfactin inhibits the growth of human colon cancer cells

To assess the inhibitory effect of surfactin on the growth of human colon cancer cells, LoVo cells were treated with surfactin at dosages ranging from 0 to 80 μM, for 12, 24, and 48 h, and MTT assay was conducted. As shown in Fig. 1, compared with untreated cells, cells treated with 80 μM surfactin for 24 and 48 h underwent almost 80% death, and 30 μM surfactin treatment for 24 h also displayed significant anti-proliferative activity. These results indicate that surfactin could suppress the proliferation of LoVo cells in dose- and time-dependent manners. In addition, two other cell lines (RAW264.7 and U937 cells) were also shown to be dose-dependently suppressed by surfactin treatment (data not shown), suggesting that the surfactin-mediated inhibition of cancer cell viability is not cell-specific. To address whether surfactin was capable of inducing apoptosis of LoVo cells, a sub-optimal concentration (30 μM), resulting in 50% inhibition at 2-day incubation was used for all further experiments.

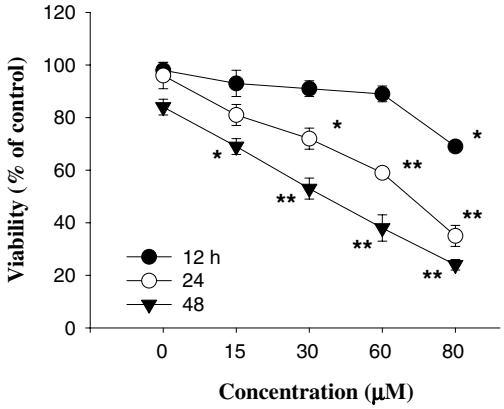

Fig. 1. Effect of surfactin on the viability of LoVo cells. LoVo cells were cultured in RPMI 1640 medium together with the indicated concentrations of surfactin for several days. The survival of surfactin-treated cells was measured by MTT assay. Data represent means ± S.E.M. of three independent observations performed in triplicate. *: $P < 0.05$ and **: $P < 0.01$ compared to normal.

3.2. Effect of surfactin on the induction of pro-apoptotic activity

To evaluate whether the surfactin-induced anti-proliferative effect was due to mediating its pro-apoptotic activity, several apoptotic parameters such as DNA fragmentation, morphological changes, and alteration of membrane polarity were chosen and carefully observed. Surfactin dose-dependently increased a characteristic DNA ladder pattern, seen in apoptotic cell death (data not shown). Clear morphological changes (nuclear condensation and the presence of perinuclear apoptotic bodies) of LoVo cells treated with surfactin (30 μM) were also observed by conventional microscopy and DAPI staining (data not shown). Furthermore, early and late-stage apoptotic cells were detected from 3 to 24 h after surfactin treatment (30 μM) (Fig. 2), according to Annexin V staining, which detects translocation of phosphatidylserine (PS), seen in the alteration of plasma membrane polarity.

3.3. Effect of surfactin on the expression of Fas/Fas ligand and the activation of caspase-3 and PARP

To study the involvement of death-receptor proteins in the surfactin-induced apoptotic process, RT-PCR analysis of Fas and its ligand was performed. The mRNA expression level of Fas receptor/ligand was increased in a dose-dependent manner, while there was no significant change of Bax expression (Fig. 3A). To demonstrate their involvement in surfactin-mediated apoptosis, two key molecules (cleaved caspase-3 and PARP, a target of caspase-3) in downstream caspase-3 events were determined. As shown in Fig. 3B, the cleaved level of caspase-3 in LoVo cells was increased dose-dependently by surfactin exposure. PARP-cleaved fragment (85 kDa) was also seen after surfactin treatment, similarly.

3.4. Effect of surfactin on the regulation of cell cycle and the expression level of cell cycle regulatory proteins

To address whether surfactin was able to arrest the cell cycle of LoVo cells, flow cytometric analysis was employed after PI staining. As shown in Fig. 4A and B, surfactin increased G_1 phase cells up to 10% and, in agreement, the number of cells in S phase also decreased. Continuously, we next analyzed the molecular alteration of cell cycle regulatory proteins at the transcriptional level. As shown in Fig. 4C, surfactin treat-