SHORT COMMUNICATION

Role of interleukin-18 on modulation of cell proliferation

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Abstract

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Interleukin-18 (IL-18), a modulator of the immune system, has been shown to be involved in several immune imbalance disorder episodes, including infections, autoimmunity and cancers. Its anti-cancer activity is mediated by the activation of NK and T cells and by the induction of IFN-γ production; however, its contribution to cancer pathogenesis has not been defined. In this study, with *in vitro* experimentation, we found that this cytokine can act as a growth factor for breast cancer (MCF-7) and colon cancer (Caco-2) cell lines as the cell number in treatment groups were significantly increased ($p<0.05$) compared to that of control groups. Interestingly, it exerts anti-proliferative properties on oral carcinoma (KB) and embryonic lung fibroblast (HEL) cell lines as a lower cell number was observed in treatment groups compared to the controls ($p<0.05$). Findings from this study demonstrated the direct interaction between this cytokine and cell proliferation and may implicate the future use of this cytokine as an immunotherapy for cancers.

Key words : interleukin-18, proliferation, cytotoxicity, anti-tumor, growth factor

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บทคัดย่อ

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บทบาทของ interleukin-18 ในการควบคุมการเพิ่มจำนวนของเซลล์

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Interleukin-18 (IL-18) เป็นซัยโตคัยน์ที่มีความสำคัญในการส่งเสริม และควบคุมการตอบสนองทางภูมิคุ้มกัน และมีความสำคัญเกี่ยวข้องกับโรคที่เกิดจากความผิดปกติทางระบบภูมิคุ้มกันหลายโรค เช่น การติดเชื้อ ภาวะออโตอิมมูนและมะเร็ง สมบัติการต้านมะเร็งของซัยโตคัยน์นี้ขึ้นกับการกระตุ้น NK และ T cells และการกระตุ้นให้มีการผลิต IFN-γ เพิ่มมากขึ้น อย่างไรก็ตาม หน้าที่ในการก่อพยาธิสภาพของมะเร็งยังไม่มีการศึกษา ในงานวิจัยนี้ผู้วิจัยได้ทดสอบฤทธิ์ของ IL-18 ต่อการเพิ่มจำนวนเซลล์ และพบว่า IL-18 สามารถเป็น Growth factor ของเซลล์มะเร็งเต้านม (MCF-7) และเซลล์มะเร็งลำไส้ (Caco-2) โดยปริมาณของเซลล์ดังกล่าวในกลุ่มที่ให้ IL-18 มีมากกว่าในกลุ่มควบคุม ($p<0.05$) นอกจากนี้ ยังพบว่า IL-18 ยังออกฤทธิ์ในการต้านการเพิ่มจำนวนเซลล์ทำให้ปริมาณเซลล์ลดลงอย่างมีนัยสำคัญ ($p<0.05$) ของเซลล์มะเร็งช่องปาก (KB) และเซลล์ไฟโบรบลาส (HEL) ผลงานวิจัยชิ้นนี้ได้แสดงให้เห็นอย่างชัดเจนถึงปฏิกิริยาระหว่าง IL-18 กับการเพิ่มจำนวนเซลล์ ซึ่งน่าจะเป็นประโยชน์ต่องานวิจัยในอนาคตเพื่อการนำซัยโตคัยน์นี้ไปใช้เพื่อ Immunotherapy ของโรคมะเร็ง

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IL-18 is a pleiotropic inflammatory cytokine that is produced from both immune and non-immune cells. It shares several properties with a pro-inflammatory cytokine interleukin-1 (IL-1), therefore categorized as a member of IL-1 family. IL-18 was first discovered as a potent IFN-γ-inducing factor in the serum and livers of mice exposed to *Propionibacterium acnes* and LPS (Nakanishi *et al*., 2001). The known activators of IL-18 production are LPS, IL-1, IL-6, TNF-α and interferons. IL-18 exerts its biological effect via its receptor complex. IL-18 receptor (IL-18R) comprises IL-18Rα which is identical to IL-1R-related protein (IL-1Rrp) and a signal transducing subunit IL-18Rβ. Signal transduction in IL-18 displays similarities to IL-1 signal transduction. The binding of IL-18 to its receptor results in the increased activation of transcription factor NF-κB (Sims, 2002), AP-1, MAPK, STAT3 and stress kinase p38 pathways (Kalina *et al*., 2000).

IL-18 modulation of immune responses is shown in both innate (Akira, 2000) and specific immunity. The ability of IL-18 in activating neutrophils and NK cells has been recently studied (Hyodo *et al*., 1999; Leung *et al*., 2001). IL-18 synergizes with IL-12 to induce IFN-γ production by T cells (Yoshimoto *et al*., 1998), and it can synergize with IL-10 to induce IFN-γ production by NK cells (Cai *et al*., 1999). In addition to its ability to induce the production of IFN-γ, IL-18 can also act as a chemoattractant of T cells (Komai-Koma *et al*., 2003).

With its primary function in activation of NK cells and induction of IFN-γ production by T cells, these made IL-18 a potential player in modulating the immune system to destroy cancer cells as has been shown in several recent studies (Golab, 2000). In an *in vivo* model of fibrosarcoma in mice, administering adenovirus vector containing gene for IL-18 into the animal could induce cancer regression and this effect could be amplified by co-infection with dendritic cells (Tanaka *et al*., 2002). IL-18 and nitric oxide synthase inhibitor synergistically enhance antitumor activity in colon carcinoma model in rat (Hegardt *et al*., 2001). These findings suggest that antitumor effect *in vivo* studies seems to be indirect to cancer cells, by stimulation of the immune system. However, the direct effect of this cytokine on cancer cell proliferation has not been defined. Therefore, in this

study, we proposed to determine if IL-18 has a regulatory role in proliferation of some cancer cell lines especially in breast cancer, oral carcinoma, and colon cancer cell lines.

Materials and Methods

Cell lines

MCF-7 is an adenocarcinoma of mammary gland. KB cell line is an epidermal carcinoma cell line derived from oral cavity. Caco-2 cell line is a colorectal adenocarcinoma cell line. These cell lines were kindly provided by Dr. Sithichai Khuntongkaew, Faculty of Dentistry, Thammasat University. Human embryonic lung fibroblast cell line (HEL) was kindly provided by the Virology Lab Unit, National Institute of Health, Department of Medical Science, Ministry of Public Health, Thailand. All cell lines were maintained in MEM (Biochrom AG Seromed®, Berlin, Germany) supplemented with 10% FBS and antibiotics (100 unit/mL of penicillin G, 100 µg/mL of streptomycin sulfate, and 0.25 µg/mL of amphotericin B) (Life Technologies, Gaithersburg, MD) and cultured at 37ºC in 5% CO_2.

Cell proliferation assay (MTT Assay)

The assay is designed for a spectrophotometric quantitation of cell growth and viability (Hansen *et al*., 1989) as well as the assessment of cytotoxicity (Carmichael *et al*., 1987). Cells (10,000 cells/well) were cultured in collagen I (50 µg/mL) coated 96-well culture plates, in a total volume of 200 µL medium with 1% FBS. The cells were cultured in the presence of recombinant human IL-18 (Peprotech Inc., Rocky Hill, NJ) (0-100 ng/mL). Both treatment and control groups were performed in 6-8 replicate wells. The relative number of viable cell was determined at 72 hours incubation, by incubating the cells with 1 mg/mL of 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, MTT, for 4 hours. The live cells utilized MTT resulting in the accumulation of formazan crystals, which were then solubilized with acid isopropanol (90% isopropyl alcohol, 0.004 N HCL) for 1 hour. The optical density of the solution was then measured at 595 nm. The absorption value of the solution directly correlates to the cell numbers. These experiments were repeated at least three times to assure the reproducibility. One-way ANOVA was used to analyze data.

LDH detection protocol

LDH release from cells was measured at the end of the proliferation experiments. Briefly, culture plates were spun at 1200 x g for 15 minutes at room temperature to ensure accumulation of all cells at the bottom of the wells. The cell-free culture media (100 µL) was collected and then incubated with 100 µL of reaction mixture (Boehringer Mannheim, Indianapolis, IN) for 30 minutes at room temperature in the dark. 1 N HCl (50 µL) was then added into each well to stop the enzymatic reaction. The optical density of the solution was then measured by using ELISA plate reader with 490 nm filter. The absorption value of the solution directly correlates to the LDH release. One-way ANOVA was used to analyze data.

Results

The overall purpose of this study was to investigate role of IL-18 on modulation of proliferation of several cell types, including both cancer and non-cancer cell lines, as this inflammatory cytokine has become an interesting target to use for cancer treatment in a form of adjuvant or as an immunopotentiator. Cell lines available for this study were MCF-7, KB, Caco-2 and HEL. MCF-7 proliferative response to IL-18 is shown in Figure 1. MCF-7 (10,000 cell/s/well) were plated in 96-well plates coated with collagen-I and incubated for 72 hours in the presence of increasing concentration of recombinant human IL-18 (0.01-100 ng/mL) in medium that contained 1% FBS. After incubation, cell number (Figure 1A) was measured by MTT assay. Data are presented as mean absorbance (λ = 595 nm) ± SEM (n = 6-8), which is proportional to the number viable cells in each group. As low as 10 pg/mL, IL-18 could stimulate MCF-7 proliferation by 30% compared to the

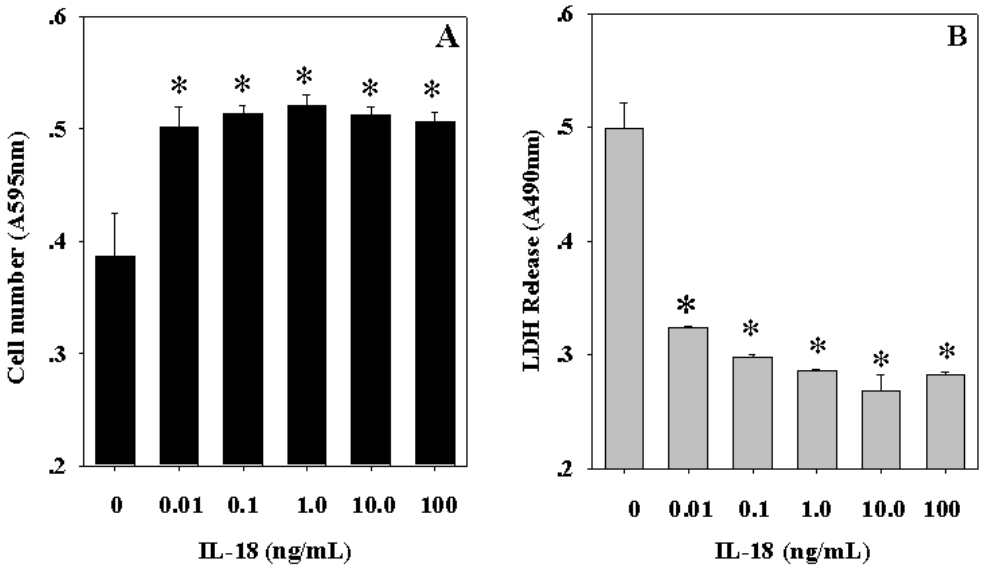

Figure 1. MCF-7 proliferative response to IL-18. MCF-7 cells were cultured in medium containing 1% FBS with increasing concentrations of IL-18 for 72 hours. Cell number (A) was measured by MTT assays and LDH release (B) was measured by LDH assays. Asterisks (*p<0.05) represent the significant different comparison against control group analyzed by one way ANOVA.

control groups (*p<0.05). However, higher concentration of the cytokine did not result in higher cell number than at concentration of 10 pg/mL.

In related study, cytotoxic effects of IL-18 on MCF-7 cells was further determined by measuring release of cytosolic lactate dehydrogenase, as shown in Figure 1B. LDH concentrations in conditioned media are proportional to cell death. Data are presented as mean absorbance (λ = 490 nm) ± SEM (n = 6-8). IL-18 treatment in these cells led to the decrease of LDH release (*p<0.05), indicating this cytokine has no cytotoxic activity on MCF-7 cells. As low as 10 pg/mL, IL-18 could inhibit LDH release by 35%. This finding indicated that IL-18 could act as a growth regulator for MCF-7 cells by inhibiting cell death.

In KB cells, as shown in Figure 2, IL-18 treatment in these cells resulted in suppression of cell proliferation (Figure 2A) in a dose dependent fashion. As low as 10 ng/mL, IL-18 could decrease cell number by 10% compared to the control group (*p<0.05). At highest concentration of cytokine used in this study, the cell number decreased by 17%. In addition, there was no change in LDH level (Figure 2B), suggesting that inhibition of cell proliferation could not be mediated by the induction of cell death. Data from this study clearly indicated that IL-18 could act as a growth regulator for KB cells.

Proliferative response of Caco-2 cell to IL-18 is shown in Figure 3. With the cytokine treatment, number of cells (Figure 3A) was significantly upregulated compared to the control group (*p<0.05). As low as 10 pg/mL of IL-18, proliferation of these cells reach the maximum with 22% increase of cell number. In the subsequent experiment, LDH release (Figure 3B) was not affected by this inflammatory cytokine, indicating that IL-18 does not induce cytotoxicity in these cells. Findings in this experiment clearly indicated that IL-18 could act as a growth regulator for Caco-2 cells.

When non-cancerous HEL cells were cultured in the medium containing increasing concentration of IL-18, proliferation (Figure 4A) of these cells could be significantly inhibited by

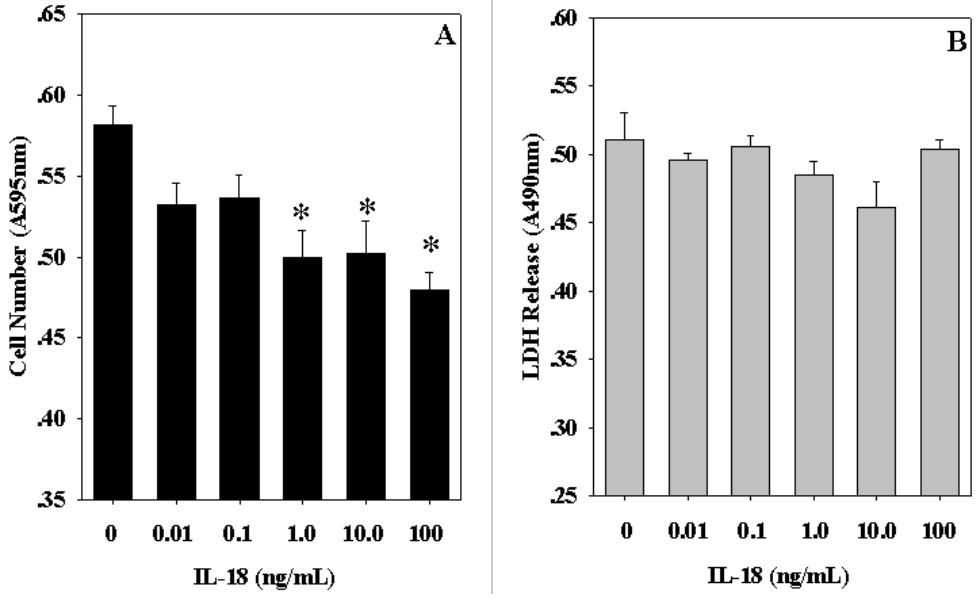

Figure 2. KB proliferative response to IL-18. KB cells were cultured in medium containing 1% FBS with increasing concentrations of IL-18 for 72 hours. Cell number (A) was measured by MTT assays and LDH release (B) was measured by LDH assays. Asterisks (*p<0.05) represent the significant different comparison against control group analyzed by one way ANOVA.

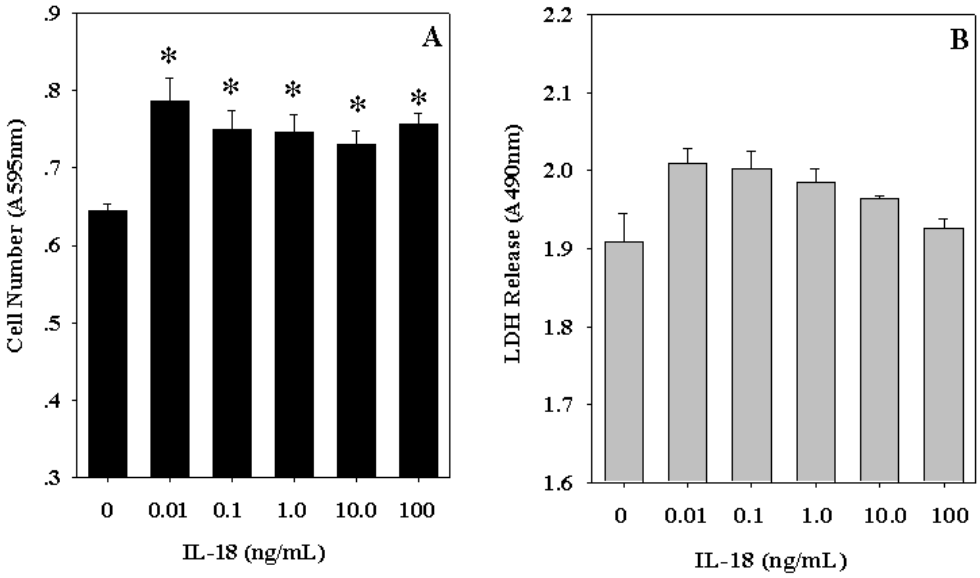

Figure 3. Caco-2 proliferative response to IL-18. Caco-2 cells were cultured in medium containing 1% FBS with increasing concentrations of IL-18 for 72 hours. Cell number (A) was measured by MTT assays and LDH release (B) was measured by LDH assays. Asterisks (*p<0.05) represent the significant different comparison against control group analyzed by one way ANOVA.

this cytokine, in dose dependent manner. As low as 10 pg/mL, this cytokine could suppress proliferation by 6% (*p<0.05) compared to the control group. With 1.0 ng/mL of IL-18, the cell number was 18% less than of control group. In addition, LDH release (Figure 4B) in these cells treated with IL-18 was increased and reach the maximum (with 11% increase) at 10 ng/mL of IL-18, suggesting that induction of cytotoxicity or cell death could attribute to the decrease of cell number.

Discussion

Not only is IL-18 a mediator of cell communication in the immune system, but this cytokine also involves in several disorder episodes. Its ability to drive T cell response to Th1 or Th2 profile is critical to development of a new avenue for therapeutic intervention for immunological imbalance diseases such as infections and cancers. Although IL-18 has been previously shown to associate with some cancer types, its function in pathogenesis or anti-tumor activity in particular cancer is still unclear. In this project, we employed an *in vitro* study to demonstrate, for the first time, the interaction between this mediator of the immune system and three cancer cell lines and one normal cell line. We demonstrated that this cytokine is a growth factor for MCF-7 (Figure 1) and Caco-2 (Figure 3) cell lines, while it can act as a growth suppressor for KB (Figure 2) and HEL (Figure 4) cell lines.

Recently, IL-18 has been shown to be involved in breast cancer. Patients with breast cancer display high serum IL-18 compared to healthy persons. It was suggested that this cytokine could be a molecular marker for diagnosis and monitoring, though its function in pathogenesis was still unknown (Merendino *et al*., 2001; Gunel *et al*., 2002). Interestingly, it was found that serum level of IL-18 is related to breast tumor size (Gunel *et al*., 2003). In addition, IL-18 may also play an important role in pathogenesis of Crohn's disease, colitis and colon cancer (Pages *et al*., 1999;

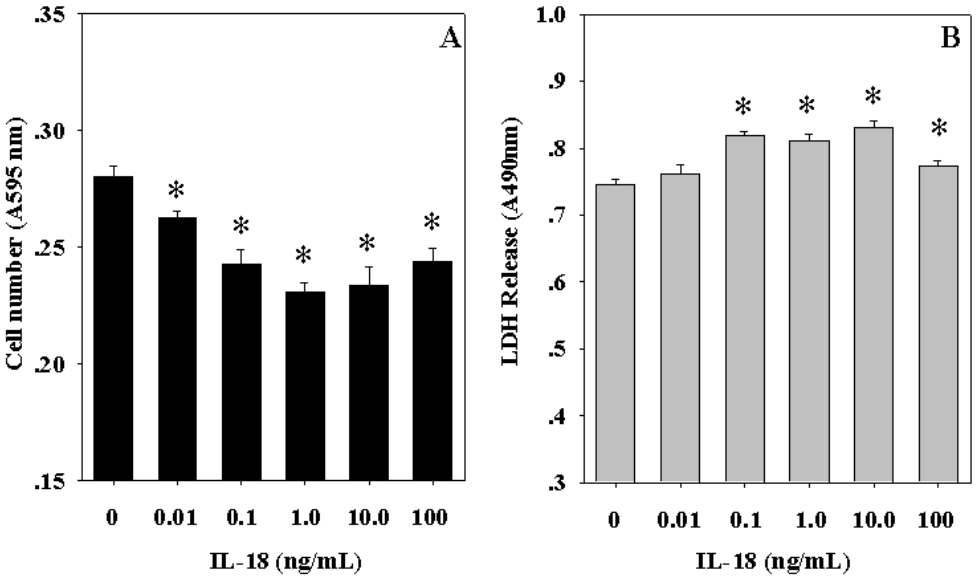

Figure 4. HEL proliferative response to IL-18. HEL cells were cultured in medium containing 1% FBS with increasing concentrations of IL-18 for 72 hours. Cell number (A) was measured by MTT assays and LDH release (B) was measured by LDH assays. Asterisks (*p<0.05) represent the significant different comparison against control group analyzed by one way ANOVA.

Paulukat *et al*., 2001). Findings from our study reveal that this cytokine can act as a growth factor for breast cancer (MCF-7) and colon cancer (Caco-2) cell lines *in vitro* and may partly suggest a possible role of IL-18 in stimulation of tumor growth.

The involvement of IL-18 in human oral carcinoma has been demonstrated in several studies. Patients with oral carcinoma often display high antibody function and high level of Th2 cytokines from advanced stage tumor (Agarwal *et al*., 2003). IL-18 has been shown to be produced by oral epithelia and carcinoma and this cytokine may function to enhance IFN-γ production by NK cells (Moore *et al*., 2003). Furthermore, serum levels of IL-18 as well as other Th1 cytokines are up-regulated in nude mice bearing salivary adeno-carcinoma treated with streptococcal product resulting in a significant reduction in tumor growth (Okamoto *et al*., 2002). For the first time, findings from our studies showed that this cytokine exerts its direct effect on inhibiting proliferation of KB cell line. Yet, its mechanisms have not been elucidated. Collectively, these findings suggest that IL-18 can be a novel antineoplastic cytokine. In addition, this cytokine is shown to have a regulatory role on proliferation of fibroblast cells. Our result showed that IL-18 has an inhibitory effect on proliferation of HEL cells. Its anti-tumor effect has also been observed in *in vivo* model of fibro-sarcoma (Cao *et al*., 1999), however; this cytokine has no effect on proliferation of fibroblast-like synoviocyte (Moller *et al*., 2002). Collectively, findings from this study demonstrate the direct interaction between this cytokine and cell proli-feration and may implicate the future use of this cytokine as an immunotherapy for cancers.

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