Apigenin Increases Natural Killer Cytotoxicity to Human Hepatocellular Carcinoma Expressing HIF-1α through High Interaction of CD95/CD95L

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Natural killer (NK) cell activity is more attenuated in hepatocellular carcinoma (HCC) patients than normal. Hypoxic-inducible factor (HIF)-1α is highly expressed in tumors to maintain their metabolism in a hypoxic environment. The expression of HIF-1α in cancers can lead to cell growth, proliferation, invasion/metastasis and immune escape. Although apigenin, a flavonoid, is known to have various biological activities, it has not been demonstrated in NK cell immune activity in HCC cells. In this study, NK-92 cells were directly cocultured with HCC SK-Hep1 cells for 24 h to evaluate NK cell activity in HCC cells or HCC cells expressing HIF-1α by apigenin. NK cell cytotoxicity to HCC cells expressing HIF-1α was significantly increased, and NK cell-activating receptors, NKG2D, NKp30 and NKp44 were highly expressed. The activating effect of apigenin on NK cells substantially induced apoptosis in HCC cells expressing HIF-1α through high expression of CD95L on the surface of NK-92 cells. Moreover, apigenin excellently inhibited the level of TGF-β1 in a coculture of NK cells and HCC cells. In conclusion, apigenin seems to be a good compound that increases NK cell cytotoxicity to HCC cells by controlling HIF-1α expression.

Keywords: Apigenin, NK, HCC, HIF-1α, CD95L (FasL; CD178)
immunomodulator of NK cells through an increase in NK proliferation by apigenin [20]. The anticancer or antimetastatic effects of apigenin in various cancers have been reported in many studies, but its effect on NK activity in cancers is not yet proved. Therefore, we tried to evaluate the effect of apigenin on NK activity in a coculture of HCC cells or HCC cells expressing HIF-1α. In this study, we found that apigenin enhanced NK cytotoxicity to HCC cells through high expression of CD95L on the surface of NK cells.

Materials and Methods
Specimen Preparation
Apigenin (≥95% pure) and cobalt chloride (CoCl₂) were purchased from Sigma-Aldrich, Inc. (USA). Apigenin and CoCl₂ were dissolved in sterile water, diluted in complete media and filtered to a 0.2 μm pore size. To induce the expression of HIF-1α in cells, CoCl₂ was used at a concentration of 250 μM.

Cell Lines and Culture
Both NK cell line NK-92 and HCC cell line SK-Hep1 were obtained from American Type Culture Collection (ATCC, USA). NK-92 cells were cultured in alpha-MEM (Gibco, USA) with the addition of 20% fetal bovine serum (FBS; Gibco), 100 U/ml penicillin and streptomycin (Gibco), 0.1 mM 2-mercaptoethanol (Sigma Aldrich), and rIL-2 (200 U/ml, BioLegend, USA). SK-Hep1 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Gibco) with 10% FBS (Gibco) and 100 U/ml penicillin and streptomycin (Gibco). All were maintained at 37°C in a humidified atmosphere with 5% CO₂.

NK Cell Proliferation Using CCK-8 Assay
The NK cell viability on apigenin was examined by Cell Counting Kit (CCK)-8 assay (Dojindo, Japan). Briefly, NK cells were plated at a density of 5 × 10⁴ cells/well with various concentrations of apigenin (0, 12.5, 25, 50, 100 and 200 μM) in a 96-well flat bottom plate and incubated for 24 h. After incubation, the cells were treated with CCK-8 solution for 3 h and then measured at the absorbance of 450 nm using a microplate reader.

NK Cytotoxic Effect on Target Cells by LDH-Release Assay
The NK-92 cytotoxic effect on target cells was identified by a CytoTox96 Non-Radioactive Cytotoxicity Assay Kit (Promega, USA). Briefly, target (SK-Hep1) cells were plated at 5 × 10³ cells each in a 96-well flat bottom plate, incubated overnight for 18-24 h and then cocultured with NK-92 cells with or without apigenin treatment (50 μM) for 24 h. A concentration of apigenin was used as previously described [20]. After 24 h, the supernatants were transferred to each well of a fresh 96-well plate, incubated in CytoTox96 reagent for 30 min, and then stop solution was added. The absorbance was assessed at 490 nm within 1 h using a microplate reader.

Surface Proteins of NK Cells Cocultured with Target Cells Using Flow Cytometry
Surface proteins of NK cells were identified using flow cytometry analysis. Briefly, target cells were plated at a density of 5 × 10⁵ cells in a 6-well plate and incubated overnight. After incubation, the cells were cocultured with NK-92 cells at 1 × 10⁶ cells for 24 h. The cells were then harvested, stained with anti-CD178-PE, anti-NKG2D-PE, anti-NKp30-PE, anti-CD56-APC, and VP for 30 min, and detected by flow cytometry (Novocyte Flow Cytometer, ACEA Biosciences, USA). All fluorescence antibodies were purchased from BD Biosciences Inc. The positive population of NK cells was analyzed within a range of anti-CD56-APC, and the value to each antibody was compensated by comparison of each emission.

Western Blot Analysis
Protein expression was examined using Western blot analysis. All protein specimens were extracted from cells cocultured by protein extraction buffer (Intron, Korea). The extracted proteins were quantified using the Bradford (Coomassie blue) assay (Gendepot, USA) and then separated by electrophoresis, transferred to polyvinylidene fluoride (PVDF) microporous membranes (Millipore, USA), and blotted with first and second antibodies. The membranes were soaked in an enhanced chemiluminescent detection solution and then visualized under Chemi-doc (Millipore). First antibodies: anti-HIF-1α (Cell Signaling, USA), granzyme-B (Santa Cruz Biotechnology, USA), anti-caspase (or cleaved caspase)-3,7,8,9 (Cell signaling), anti-Smac (Santa Cruz Biotechnology), anti-XIAP (Santa Cruz Biotechnology), anti-FADD (Santa Cruz Biotechnology), anti-Fas (Santa Cruz Biotechnology), and anti-GAPDH (Santa Cruz Biotechnology).

ELISA Assay
The quantitation of cytokines was examined by BD OptEIA Set Human IFN-γ, TGF-β1, IL-10 (BD Bioscience) and Human ELISA Kit IL-18 and IL-21 (Invitrogen, USA). Briefly, target cells were cultured in a 6-well plate overnight and cocultured with effector cells (NK-92, ratio of E:T = 2:1) for 24 h with or without apigenin treatment (50 μM). After the coculture, the supernatants were harvested for ELISA assay. All experiments were conducted according to the manufacturer’s instructions. The absorbance of IFN-γ, TGF-β1 and IL-10 was measured at 450 nm with λ correction at 570 nm, and the absorbance of IL-18 and IL-21 was measured at 450 nm with λ correction at 620 nm using a microplate reader (BMG Labtech, Germany).
**Statistical Analyses**

All data were analyzed using Microsoft Excel. The results were presented as means ± SD, and the comparison of several means was performed by one-way or two-way analysis of variance followed by Fisher's exact test. Differences between groups at a *p*-value of less than 0.05 were considered significant.

**Results**

**Increase in NK Cytotoxicity through High Secretion of Granzyme B from NK Cells to HIF-1α-Expressing HCC Cells by Apigenin**

To identify the increase in NK cytotoxicity to HCC cells by apigenin by LDH-release assay, a secretion of perforin and granzyme from NK cells was examined in a coculture of HCC cells with or without apigenin treatment (50 μM). The concentration of apigenin used in NK cytotoxicity to HCC cells was examined by CCK-8 assay. As shown in Fig. 1A, apigenin did not have a negative effect on NK cell viability while seeming to increase cell proliferation at over 50 μM (*p < 0.05). NK cytotoxicity to target cells showed no change with apigenin treatment (Fig. 1B; control; 30.96%, apigenin; 28.87%, ns). The secretion of GrzB from NK cells to target cells also showed no significant difference between control and apigenin treatment (Fig. 1C, ns). However, apigenin interestingly increased NK cytotoxicity and the production of GrzB in a coculture of NK and HCC cells when HIF-1α was expressed (NK cytotoxicity; Fig. 1B; CoCl2 4.68%, CoCl2+apigenin 9.99%; *, **p < 0.05).

**Increased Expression of NK-Activating Receptors NKG2D, NKp30 and NKp44 on the Surface of NK Cells in Coculture of HIF-1α-Expressing HCC by Apigenin**

NK cell activity depends on a balance between NK-activating and inhibitory receptors on the cell surface. Thus, we identified the expression of human NK-activating receptors on the surface of NK-92 cells cocultured with HCC with or without treatment with CoCl2 (250 μM) and apigenin (50 μM). NK-activating receptors NKG2D, NKp30 and NKp44 were examined by staining of fluorescence antibodies (anti-NKG2D-APC, anti-NKp30-PE, and anti-NKp44-PE) using flow cytometry. In Figs. 2A-2C, NKG2D and NKp30 were similarly expressed in NK-92 cells cocultured with HCC and apigenin-treated HCC cells (NKG2D+; non-treated 71.01%, treated 68.09%; NKp30+; non-treated 64.27%, treated 67.50%; ns); however, the expression of NK44 on NK-92 was significantly lower in a coculture of HCC treated with apigenin (NKp44+; non-treated 20.72%, treated 17.41%, *p < 0.05). Apigenin had no effect on NK cell activity against HCC cells, but interestingly, it activated NK cells toward HCC cells when HIF-1α was expressed. The expression of NKG2D, NKp30 and NKp44 was higher in a coculture of NK cells and HIF-1α-expressing HCC cells with apigenin treatment than without (Figs. 2A-2C, NKG2D+; CoCl2 18.54%, CoCl2+apigenin 24.49%, NKp30+; CoCl2 34.35%, CoCl2+apigenin 38.67%, NKp44+; CoCl2 9.49%, CoCl2+apigenin 11.2%, **p < 0.05).

**Decreased Expression of HIF-1α in HCC Cells Cocultured with NK Cells by Apigenin**

To recognize whether apigenin inhibits HIF-1α expression, we investigated the expression of HIF-1α in HCC cells cocultured with NK cells using Western blot analysis. As shown in Fig. 3A, the expression of HIF-1α in HCC cells cocultured with NK cells was significantly lowered when treated with apigenin (*, **p < 0.05).

**Higher Interaction of Fas (CD95)/FasL (CD95L; CD178) between NK Cells and HCC Cells by Apigenin**

The secretion of GrzB from NK cells to HCC cells was significantly increased by apigenin (Fig. 1C). According to recent reports, GrzB stimulates a death receptor on the surface of target cells and subsequently induces the apoptosis of target cells [12]. Thus, we tried to examine a death receptor associated with GrzB. In Figs. 3B and 3C, the expression of CD95L (FasL) was significantly increased after treatment with apigenin, in both HIF-1α- and HIF-1α-untreated HCC cells. Moreover, CD178 (CD95L) on the surface of NK-92 cells was significantly upregulated by apigenin regardless of HIF-1α expression (Fig. 3C, CD178; CoCl2 8.1%, apigenin 11.72%, CoCl2+apigenin 9.69%, **p < 0.05).
Increased CD95-Mediated Apoptosis in HCC Expressing HIF-1α by Expressing High CD95L (CD178) on the Surface of NK Cells by Apigenin

The receptor CD95 binds to CD95L, which is implicated in immune homeostasis and immune surveillance and is expressed on the surface of activated T lymphocytes and NK cells [21, 22]. Binding CD95L to CD95 stimulates increased CD95-Mediated Apoptosis in HCC Expressing HIF-1α by Expressing High CD95L (CD178) on the Surface of NK Cells by Apigenin.
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the activation of FADD, and that triggers apoptosis by the activation of caspase-8. CD95L (CD178) on the surface of NK-92 cells and CD95L (Fas) and FADD in HCC cells increased with apigenin treatment (Fig. 3C). Thus, to verify whether apigenin actually activates the caspases in HCC cells cocultured with NK-92 cells, the expression of caspases (-3,-7,-8 and -9) was investigated by Western blot analysis. The expression of cleaved caspase-8 showed no difference in HCC cells cocultured with NK-92 cells by apigenin but was significant when HIF-1α was expressed (Fig. 4C). Moreover, as shown in Figs. 4A and 4B, the effectors caspase-3 and caspase-7 were significantly activated in HCC expressing HIF-1α cocultured with NK-92 cells by apigenin. The expression of cleaved caspase-9, however, showed no difference between HCC cells in the presence or absence of apigenin (Fig. 4D).

Decrease in TGF-β1 Released from NK-92 Cells Cocultured with HCC Cells Regardless of HIF-1α Expression by Apigenin

Various studies have shown that cytokines in a tumor microenvironment could decide the function of immune cells against cancer cells [23]. Therefore, we examined cytokines secreted from a coculture of NK cells and HCC cells by apigenin treatment. Initially, we identified the level of IFN-γ in the supernatant of a coculture of NK-92 and HCC cells in the presence or absence of apigenin. Unexpectedly, the quantity of IFN-γ in the supernatant of a coculture of NK-92 cells and HCC cells significantly increased when HIF-1α was expressed by treatment with CoCl2 (250 μM) as compared with no expression of HIF-1α, and apigenin conversely rather decreased the level of IFN-γ (Fig. 5A; *, **p < 0.05). Furthermore, the expression of HIF-1α in HCC cells cocultured with NK-92 cells significantly increased the level of TGF-β; however, that was remarkably decreased by apigenin (Fig. 5B; *, **p < 0.05). The cytokines IL-18, IL-21 and IL-10, which can be correlated with NK cell activity, were not greatly affected by apigenin, albeit IL-18 decreased (Figs. 5C-5E; *p < 0.05).

Fig. 4. High induction of apoptosis in HCC cells cocultured with NK cells by apigenin. The expression of (A) cleaved caspase-3, (B) cleaved or caspase-7, (C) cleaved caspase-8, (D) cleaved caspase-9 in HCC cells cocultured with NK cells treated or not treated with CoCl2 (250 μM) and apigenin (50 μM). All data were compared at *p < 0.05 vs non-treated and **p < 0.05 vs CoCl2, and presented as means ± SD from three independent experiments.

Fig. 5. Inhibitory secretion of TGF-β1 from a coculture of NK cells and HCC cells by apigenin. Several cytokines were analyzed in the supernatants of a coculture of NK cells and HCC cells with or without treatment with CoCl2 (250 μM) and apigenin (50 μM) using ELISA assay (A) IFN-γ, (B) TGF-β1, (C) IL-18, (D) IL-21, and (E) IL-10 secreted from a coculture of NK cells and HCC cells. The data are presented as means ± SD from three independent experiments. *p < 0.05 vs non-treated, **p < 0.05 vs CoCl2.
Discussion

In this study, apigenin showed an increase in NK cell cytotoxic effect to HCC cells or HCC cells expressing HIF-1α in vitro. Various cancer types have shown a low frequency and dysfunction of NK cells, and that might promote the metastasis of cancer cells [24]. Moreover, NK cells in patients with HCC commonly present low cytotoxicity and decreased production of interferon (IFN)-γ [11].

Hypoxia is a low-oxygen (O₂ ≤ 1%) condition generated by cancer metabolism and it induces DNA damage. Hypoxia is known to cause genomic changes that tolerate poor nutrition and a hostile microenvironment in tumor cells, and thus tumors are able to survive [25]. HIF-1α is commonly expressed in many tumors, and this inhibits the expression of Bid and pro-apoptotic Bcl-2-family protein [26] and stimulates the expression of survivin, an apoptosis inhibitor [27]. Moreover, the invasion and metastasis of cancer cells is promoted by hypoxia because it can induce the expression of interleukin (IL)-6, platelet-derived growth factor (PDGF) and transforming growth factor (TGF)-β [28]. In this study, we first tried to examine the NK cell function in HCC cells when HIF-1α was expressed. HIF-1α expression between NK and HCC cells attenuated the NK cytotoxic effect to HCC cells (Fig. 1B), and also reduced the secretion of granzyme B (GrzB) from NK cells into target cells (Fig. 1C). GrzB is a unique serine protease found in the lytic granules of NK cells and T lymphocytes (CTL). NK cells recognize target cells and then secrete GrzB and perforin into the inter space between them. GrzB in the cytoplasm of target cells leads to the cleavage of caspases, which induces their apoptosis [29]. The results indicated that the expression of HIF-1α in HCC cells decreased NK cytotoxicity. In addition, the expression of NK-activating receptors NKG2D, NKp30 and NKp44 on the surface of NK cells significantly decreased with HIF-1α expression (Fig. 2), which was implicated in the decline of an apoptotic effect of NK cells on HCC cells through a decrease in the cleavage of caspase-3, -7, -8, and -9 (Fig. 4). Recently, many studies have focused on the composition of chemokines or cytokines within the tumor microenvironment. The pro-inflammatory cytokines IL-1β, TNF, and IL-6 can induce the transition of EMT in head and neck cancers [30] and anti-inflammatory cytokine IL-10 can lead to tumor cell proliferation through an induction of STAT3 activation in gastric cancer cells [31]. Also, the presence of IL-10, TGF-β, and prostaglandins in the tumor microenvironment plays a role of immune evasion that enables the anti-tumor activities of NK, T and B cells [32-34]. Thus, it is necessary to confirm the level of cytokines, especially that concerned with NK activity. IFN-γ is produced by NK cells by stimulating interleukin(IL)-2, and so we examined the level of IFN-γ produced by a coculture of NK cells and HCC cells. In a coculture of NK cells and HCC expressing HIF-α, the level of IFN-γ was significantly increased (Fig. 5A), contrary to our expectations, while the level of TGF-β1 was substantially increased as well (Fig. 5B). Recently, it has been reported that IFN-γ can lead to EMT transition in pancreatic cells [35] or endometrial cancer cells [36] through the stimulation of MUC4 transcription, which is expressed in an aggressive or metastatic tumor phenotype by the activation of STAT3 [37]. Moreover, several studies showed that IFN-γ signaling enhanced the expression of PD-L1 and induced immune suppression [38]. This evidence fits with our result that an increased level of IFN-γ might be not important in NK cytotoxicity to HCC. However, a high level of TGF-β1 seemed to impair NK activity in HCC (Figs. 1-3). In addition, the level of IL-18 significantly decreased (Fig. 5C), and this was considered to not affect the production of IFN-γ from NK cells but had an inhibitory effect on other factors such as the inflammasome in HCC cells. Our previous study showed that the expression of inflammasome (NLRP3) in HCC could attenuate NK cell cytotoxic ability through the low interaction of NKG2D-MICA/B [39]. This result could mean that a coculture of NK cells and HCC cells might change their environment to induce low NK cell activity by expressing HIF-1α.

The anticancer effect of apigenin is shown in various cancer types containing hepatocellular carcinoma SK-Hep1 and BEL-7402 [40]. According to a recent report, apigenin at a concentration of 25 μg/ml increased NK cell proliferation [20], and the NK immunomodulatory effect of apigenin was recently proved through an induction of human peripheral blood mononuclear cell proliferation [41]. In this study, we first tried to examine the NK cell function in HCC cells when HIF-1α was expressed. HIF-1α expression between NK and HCC cells attenuated the NK cytotoxic effect to HCC cells (Fig. 1B), and also reduced the secretion of granzyme B (GrzB) from NK cells into target cells (Fig. 1C). GrzB is a unique serine protease found in the lytic granules of NK cells and T lymphocytes (CTL). NK cells recognize target cells and then secrete GrzB and perforin into the inter space between them. GrzB in the cytoplasm of target cells leads to the cleavage of caspases, which induces their apoptosis [29]. The results indicated that the expression of HIF-1α in HCC cells decreased NK cytotoxicity. In addition, the expression of NK-activating receptors NKG2D, NKp30 and NKp44 on the surface of NK cells significantly decreased with HIF-1α expression (Fig. 2), which was implicated in the decline of an apoptotic effect of NK cells on HCC cells through a decrease in the cleavage of caspase-3, -7, -8, and -9 (Fig. 4). Recently, many studies have focused on the composition of chemokines or cytokines within the tumor microenvironment. The pro-inflammatory cytokines IL-1β, TNF, and IL-6 can induce the transition of EMT in head and neck cancers [30] and anti-inflammatory cytokine IL-10 can lead to tumor cell proliferation through an induction of STAT3 activation in gastric cancer cells [31]. Also, the presence of IL-10, TGF-β, and prostaglandins in the tumor microenvironment plays a role of immune evasion that enables the anti-tumor activities of NK, T and B cells [32-34]. Thus, it is necessary to confirm the level of cytokines, especially that concerned with NK activity. IFN-γ is produced by NK cells by stimulating interleukin(IL)-2, and so we examined the level of IFN-γ produced by a coculture of NK cells and HCC cells. In a coculture of NK cells and HCC expressing HIF-α, the level of IFN-γ was significantly increased (Fig. 5A), contrary to our expectations, while the level of TGF-β1 was substantially increased as well (Fig. 5B). Recently, it has been reported that IFN-γ can lead to EMT transition in pancreatic cells [35] or endometrial cancer cells [36] through the stimulation of MUC4 transcription, which is expressed in an aggressive or metastatic tumor phenotype by the activation of STAT3 [37]. Moreover, several studies showed that IFN-γ signaling enhanced the expression of PD-L1 and induced immune suppression [38]. This evidence fits with our result that an increased level of IFN-γ might be not important in NK cytotoxicity to HCC. However, a high level of TGF-β1 seemed to impair NK activity in HCC (Figs. 1-3). In addition, the level of IL-18 significantly decreased (Fig. 5C), and this was considered to not affect the production of IFN-γ from NK cells but had an inhibitory effect on other factors such as the inflammasome in HCC cells. Our previous study showed that the expression of inflammasome (NLRP3) in HCC could attenuate NK cell cytotoxic ability through the low interaction of NKG2D-MICA/B [39]. This result could mean that a coculture of NK cells and HCC cells might change their environment to induce low NK cell activity by expressing HIF-1α.

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Taken together, NK cytotoxic effect on HCC cells was dramatically attenuated by expressing HIF-1α. In

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particular, the expression of HIF-1α between NK and HCC cells significantly decreased secretion of GrzB from NK cells leading to a decreased killing effect of NK cells toward HCC cells. The attenuation of the NK cell cytotoxic effect on HCC cells expressing HIF-1α, however, could be restored by apigenin. Treatment with apigenin in a coculture of NK and HCC under HIF-1α expression increased the NK cytotoxic effect but had no effect when HIF-1α was not expressed. Interestingly, apigenin increased all expressions of CD178 (CD95L;Fas) on the surface of NK cells in a coculture of HCC cells or HCC cells expressing HIF-1α; however, a difference in the increase in the activation of caspases and GrzB was shown when HIF-1α was expressed. In conclusion, apigenin can be a good compound to increase the NK cytotoxic effect on HCC by inducing high expression of CD95L on the surface of NK cells in a hypoxic condition.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.
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