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Licochalcone A is a potent inhibitor of TEL-Jak2-mediated transformation through the specific inhibition of Stat3 activation

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ABSTRACT

Aberrant activation of Jak/Stat signaling causes a number of hematopoietic disorders and oncogenesis, and therefore the effective inhibitors of the Jak/Stat signaling pathway may be therapeutically useful. TEL-Jak2 gene fusion, which has been identified in human leukemia, encodes a chimeric protein endowed with constitutive tyrosine kinase activity. Expression of TEL-Jak2 protects Ba/F3 cells from IL-3 withdrawal-induced apoptotic cell death and leads to IL-3-independent growth. However, its mechanisms remain to be only partially understood. Here, we first found that Licochalcone A, one of the flavonoids isolated from the root of *Glycyrrhiza inflate*, inhibited TEL-Jak2-mediated cell proliferation and survival in the absence of IL-3. Licochalcone A failed to inhibit the activity of TEL-Jak2, however, this induced apoptosis of TEL-Jak2-transformed cells with a much lower concentration in the absence of IL-3 than in the presence of IL-3. Interestingly, Licochalcone A significantly inhibited the phosphorylation and nuclear localization of Stat3, which is essential for TEL-Jak2-induced cell transformation. These data suggest that Licochalcone A is a specific inhibitor for Stat3 and would be employed for the treatment of various diseases caused by disorders of the Jak/Stat pathway.

1. Introduction

The tyrosine kinase, Jak2, regulates various cytokine signaling pathways by binding to hematopoietic cytokine receptors [1,2]. In the normal cells, Jak2 is maintained as its inactive form until the receptors linking Jak2 are activated. Once cytokines bind to their specific receptors, the associated Jak2 is rapidly phosphorylated within the activation

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Abbreviations: DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; MAP kinase, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; IL-3, interleukin-3; Jak, Janus kinase; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor kappaB; NP-40, Nonidet P40; PBS, phosphate buffered saline; PI3-kinase, phosphoinositide 3-kinases; PVDF, polyvinylidene difluoride; Stat, signal transducers and activators of transcription.

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loop (Y^{1007}–Y^{1008}) and activated by promoting conformational change [3,4].

Jak kinases are characterized by the presence of seven regions of sequence similarity, designated as Janus homology (JH) domains and these JH domains are termed as JH1–JH7 [5]. JH1 contains a catalytically active tyrosine kinase domain located in the C terminus of Jak kinases. The adjacent domain, JH2 exhibits a high sequence identity with JH1 domain, but lacks the kinase activity and is termed as the pseudokinase domain. Theoretical models of the Jak2 structure suggest that the JH1 and JH2 domains face each other and the activation loop of Jak2 is likely buried in this interface [6]. Thus, cytokine-induced phosphorylation of the activation loop is believed to prevent the interaction between JH1 and JH2, and therefore relieves its inhibition [7,8].

Since Jak2 is involved in various physiological phenomena under precise regulation, the deregulation of Jak2 activation has been associated with hematopoietic disorders and oncogenesis [9,10]. One of the deregulation mechanisms of Jak2 signaling is caused by chromosomal rearrangement. The chromosomal translocation [t (9; 12) (p24; p13)] is associated with T cell childhood acute lymphoblastic leukemia and results in the production of fusion protein TEL-Jak2, which contains the Jak2 catalytic domain (JH1) and the oligomerization domain of TEL, one of the Ets transcription factor family members [11,12]. Since the presence of this oligomerization domain, TEL-Jak2 constitutively exhibits the oligomerization state. And because of this, TEL-Jak2 protein shows constitutive activation of tyrosine kinase activity leading to the derivative activation of downstream molecules, including a signal transducer and activator of transcripition-3 (Stat3), Stat5 [13,14], PI3-kinase/Akt [15,16], MAP kinases [17] and NF-κB [18].

Liquorice root has been used as a traditional medicine in the East and West for the treatment of gastric ulcer, bronchial asthma and inflammation, and is therefore expected to be an effective medicine without serious side effects. Licochalcone A is a major and biogenetically characteristic chalcone isolated from the root of *Xinjiang liquorice*, *Glycyrrhiza inflate* Batalin [19]. Previous study showed that Licochalcone A possessed radical scavenging effects [20], anti-leishmanial activity and antimicrobial activity inhibiting the growth of *Staphylococcus aureus*, *Bacillus subtilis* and the activity of *Helicobactor pylori* [21,22]. So far, we have studied the novel effects of Licochalcone A on inflammatory cytokine signaling pathways. In the process of examining the influence on the LPS signaling pathway using murine macrophage-like cells, RAW264.7, we found that Licochalcone A has a cytotoxic effect. These results prompted us to examine whether Licochalcone A exhibited anti-tumorgenesis activity.

TEL-Jak2-transformed cells can be considered as model cells for certain human leukemia and lymphomas. Thus, we analyzed the effect of Licochalcone A on TEL-Jak2-mediated transformation. Here, we showed the first evidence that Licochalcone A potently induced apoptotic death of TEL-Jak2-transformed cells. Licochalcone A was also shown to specifically inhibit TEL-Jak2-induced Stat3 activation but not Stat5 or ERK activation. Our data demonstrate the possibility that Licochalcone A can be used as a powerful anti-cancer drug with few side effects.

2. Materials and methods

2.1. *Licochalcone A and its derivatives*

To prepare Licochalcone A, Xin-jiang liquorice (300 g) was extracted with MeOH (3.0 l) for 3 days and the extracts were loaded onto a silica gel column chromatography with $CHCl_3$/MeOH (15:1). The Licochalcone A rich fraction was purified by silica gel chromatography with the gradient of *n*-hexane/EtOAc at the ratio of 3–1 and 2–1. Purified Licochalcone A (2.4 g) was then analyzed by ^1H NMR and mass-spectrometry analysis.

Compound 1 (5-(1,1-dimethylallyl)-2,4,4′-trimethoxychalcone) was synthesized by methylation of Licochalcone A. Licochalcone A solved in acetone (0.059 mmol/2.0 ml) was mixed with 0.89 mmol KOH and 0.32 mmol methyl iodine and stirred at room temperature for 14 h. Then from reaction mixture, organic layer was isolated and treated with 5% HCl solution. After dried up, compound 1 was purified by silica gel chromatography equilibrated with *n*-hexane/ethyl acetate (2:1). Purity of purified compound 1 (18.2 mg) was 83%.

Compound 2 (4,4′-diacetoxy-5-(1,1-dimethylallyl)-2-methoxychalcone) was synthesized by acetylation of Licochalcone A. Licochalcone A solved in pyridine (0.3 mmol/10 ml) was mixed with 1 ml of acetic anhydride and stirred at room temperature for 2 h. Then methanol was added and organic layer and aqueous layer were separated. Isolated organic layer was treated with 5% HCl solution. After dried up, compound 2 was purified by silica gel chromatography equilibrated with *n*-hexane/ethyl acetate (1:1) and its purity was 82%.

Compound 3 (4,4′-dicarboxymethyl-5-(1,1-dimethylallyl)-2-methoxychalcone) was obtained by carboxymethylation of Licochalcone A. Licochalcone A solved in acetone (0.3 mmol/2.0 ml) was mixed with 1.07 mmol KOH and 0.98 mmol ethyl bromoacetate and stirred at 0 °C for 2 h. Then from reaction mixture, organic layer was isolated and treated with 5% HCl solution. After dried up, compound 3 was purified by recrystallization from *n*-hexane/ethyl acetate/ethanol to give compound 4 (70 mg, 80%). Purity of obtained compound 3 (70 mg) was 70%.

Compound 4 (5-(1,1-dimethylallyl)-4-hydroxy-2-methoxychalcone) was synthesized by two steps, including aldol condensation reaction and Claisen rearrangement on heating. First, 4-hydroxy-2-methoxybenzaldehyde solved in acetone (0.66 mmol/10 ml) was mixed with 0.74 mmol prenyl bromide and 110 mg K_2CO_3. And then mixture was stirred at room temperature for 4 h. After evaporation, obtained organic layer in $CHCl_3$. This organic layer was washed with water and then dried up. First product was absorbed by silica gel chromatography equilibrated with *n*-hexane/ethyl acetate (20:1) and eluted by gradient with *n*-hexane/EtOAc (8:1). The efficiency of first reaction was 86%. Next, to a solution of acetophenone (0.23 mmol) and First product (0.45 mmol) in EtOH (10 ml), 60% of KOH solution (5 ml) was added and the reaction mixture was stand at 0 °C for 2 h. The reaction was quenched with 5% HCl and extracted with ethyl acetate. After dried up, the product of second reaction was purified by silica gel chromatography (50:1–6:1 of *n*-hexane/ethyl acetate). The efficiency of second reaction was 84%. The product of second reaction was solved in *N*,*N*-dimethyl-aniline/acetic anhydride (2:1). The mixture was stirred at 180 °C under N_2 atmosphere

for 2 days. The reaction was quenched with 5% HCl and extracted with ethyl acetate. The organic phase was washed by brine and then dried over anhydrous Na_2SO_4. The reaction efficiency of final step was 23%. The compound 4 was purified by silica gel column chromatography (20:1–6:1 of *n*-hexane/ethyl acetate). The purity of obtained compound 4 (109 mg) was 48%. The details about ^1H NMR analysis and LC-MS analysis of compound 1–4 were available in supplemental information. Licochalcone A and all derivatives were resolved up to 30 mM in DMSO.

2.2. Reagents

Recombinant IL-3 was purchased from R&D System (Minneapolis, MN, USA). Anti-phospho-Jak2 ($Y^{1007/1008}$) antibody, anti-phospho-Stat3 antibody, anti-phospho-Stat5 antibody, anti-phospho-ERK antibody, anti-phospho-JNK antibody, anti-Stat3 antibody, anti-Stat5 antibody, anti-ERK antibody, anti-JNK antibody were from Cell Signaling Technology (Danvers, MA, USA). Anti-β-actin antibody, anti-Jak2 (C-20) and anti-TEL antibody (N-19) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Peroxidase-conjugated rabbit anti-mouse and goat anti-rabbit secondary antibodies were from Dako (Glostrup, Denmark).

2.3. Cell culture and retroviral infection

The IL-3 dependent murine hematopoietic cell line Ba/F3 was cultured in RPMI-1640 (Nissui Seiyaku, Tokyo, Japan) supplemented with 10% FBS (BioWest, France), 100 μg/ml L-glutamine (Nacalai tesque, Tokyo, Japan), 100 μg/ml penicillin (Nacalai tesque), 100 μg/ml streptomycin (Nacalai tesque) and 2.5 ng/ml murine IL-3 (R&D System). Retroviruses harboring TEL-Jak2 cDNA and control retrovirus (MSCV-ires-GFP) were prepared as described previously [23]. Ba/F3 cells expressing TEL-Jak2 (Ba/F3/TEL-Jak2) and control virus-infected cells (Ba/F3/MSCV) were established by retroviral infection of parental Ba/F3 cells with the retrovirus harboring TEL-Jak2 cDNA and control virus, respectively. And then, the cells infected were sorted based on GFP expression, as described previously [23].

2.4. Ba/F3 cell growth assay

Transduced and exponentially growing Ba/F3 cells were washed twice with PBS and incubated with RPMI-1640 supplemented with 1% FBS and 100 mg/ml L-glutamine in the presence or absence of IL-3 (2.5 ng/ml) for the indicated times. Living cells were counted using a Beckman Coulter VI-Cell (Beckman Coulter, Fullerton, CA). Cell viability was checked by the trypan blue exclusion method.

2.5. BrdU cell proliferation assay

Transduced and exponentially growing Ba/F3 cells were washed twice with PBS and suspended with RPMI-1640 supplemented with 1% FBS and 100 μg/ml L-glutamine. 2×10^4 cells were left untreated or stimulated with IL-3 (2.5 ng/ml) for 24 h and then the proliferation rate was determined using the 5-bromo-2′ deoxy-uridine (BrdU) labeling and detection ELISA kit III (Roche, Mannheim, Germany).

2.6. Cell cycle analysis

After treatment, cells were washed with PBS and fixed with 70% (v/v) ethanol at −20 °C overnight. Cells were then centrifuged at 5000 r.p.m for 2 min and resuspended in PBS containing 10 μg/ml RNaseA (Wako, Tokyo, Japan) and 100 μg/ml propidium iodide (PI) (SIGMA ALDRICH, United Kingdom). Following 30 min incubation, cell cycle parameters were determined by flow-cytometory analysis using FACS Calibur. All data were recorded and analyzed using CellQuest software.

2.7. DNA fragmentation assay

Genomic DNA was prepared for gel electrophoresis as described previously [24]. Electrophoresis was performed on a 1% (w/v) agarose gel in Tris-boric acid buffer. Fragmented DNA was visualized by staining with ethidium bromide after electrophoresis.

2.8. Transfection of siRNA

Control siRNA and siRNA targeting murine Stat3 were purchased from Dharmacon (GE Healthcare UK Ltd., United Kingdom). siRNA was transfected using DarmaFECT, according to the manufacturer's instructions. Cell lysates were prepared with 48 h post-transfection and analyzed by Western blotting.

2.9. Immunoprecipitation and Western blotting

Cells were harvested in ice-cold PBS and lysed in NP-40 lysis buffer (50 mM Tris–HCl pH 7.4, 10% glycerol, 50 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 20 mM NaF, 0.2 mM Na_3VO_4) supplemented with protease inhibitors. Cell lysates were centrifuged at 15,000 r.p.m for 15 min to remove debris, and the supernatants were incubated with the indicated antibody for 4 h. Immune complexes were precipitated with protein G-Sepharose (Zymed Laboratory, South San Francisco, CA), washed three times with lysis buffer, and then eluted with sample buffer for SDS-PAGE. Eluted proteins were resolved by SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA). Membranes were probed using the designated antibodies and visualized with the ECL detection system (GE Healthcare UK Ltd.).

2.10. In vitro kinase assay

The cells were lysed in kinase lysis buffer (10 mM Tris–HCl (pH 7.5), 1% Triton X-100, 20% glycerol, 5 mM EDTA, 50 mM NaCl, 50 mM NaF and 1 mM Na_3VO_4) supplemented with protease inhibitors. TEL-Jak2 was immunoprecipitated using anti-TEL antibody and washed three times with kinase lysis buffer and twice with kinase assay buffer (10 mM HEPES (pH 7.4), 50 mM NaCl, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 50 mM NaF, 1 mM Na_3VO_4). The immunocomplex was resuspended in 50 μl of kinase buffer containing 1 μCi of [γ-^{32}P] ATP (Amersham), 50 ng of Jak2 peptide (VLPQDKEYYKVKEPGES) and incubated at 30 °C for 30 min. An aliquot of reaction mixture was transferred onto p81 phosphocellulose paper (Whatman) and then the p81 phosphocellulose paper was washed three times for 5 min

each with 0.75% phosphoric acid and once for 5 min with acetone. The radioactivity was measured using scintillation counter. The counts detected in the absence of peptide were used as a back ground control as described previously [23].

3. Results

3.1. *Expression of TEL-Jak2 in Ba/F3 cells drives cytokine-independent growth and inhibits IL-3 withdrawal-induced apoptotic cell death*

TEL-Jak2 is the gene product obtained form translocation of the Jak2 gene with the translocated Ets Leukemia gene *TEL*. The fusion protein, TEL-Jak2, contains the N-terminal region of TEL, which comprises the oligomerization domain and the JH1 (kinase) domain of Jak2. To investigate the function of TEL-Jak2, we established Ba/F3 cells expressing TEL-Jak2 (Ba/F3/TEL-Jak2 cells), as shown in Fig. 1A, and assessed the requirement for IL-3 to support proliferation (Fig. 1B). As in previous reports [11,12,25], the expression of TEL-Jak2 conferred growth factor independence on BaF3 cells (Fig. 1B left). In the presence of IL-3, no differences in proliferation were seen in parental Ba/F3 cells, control virus-infected Ba/F3 cells (Ba/F3/MSCV cells) and Ba/F3/TEL-Jak2 cells (Fig. 1B right). Cytokine deprivation has been classically used to study the molecular processes of apoptosis. Following IL-3-withdrawal, Ba/F3 cells and Ba/F3/MSCV cells underwent cell death.

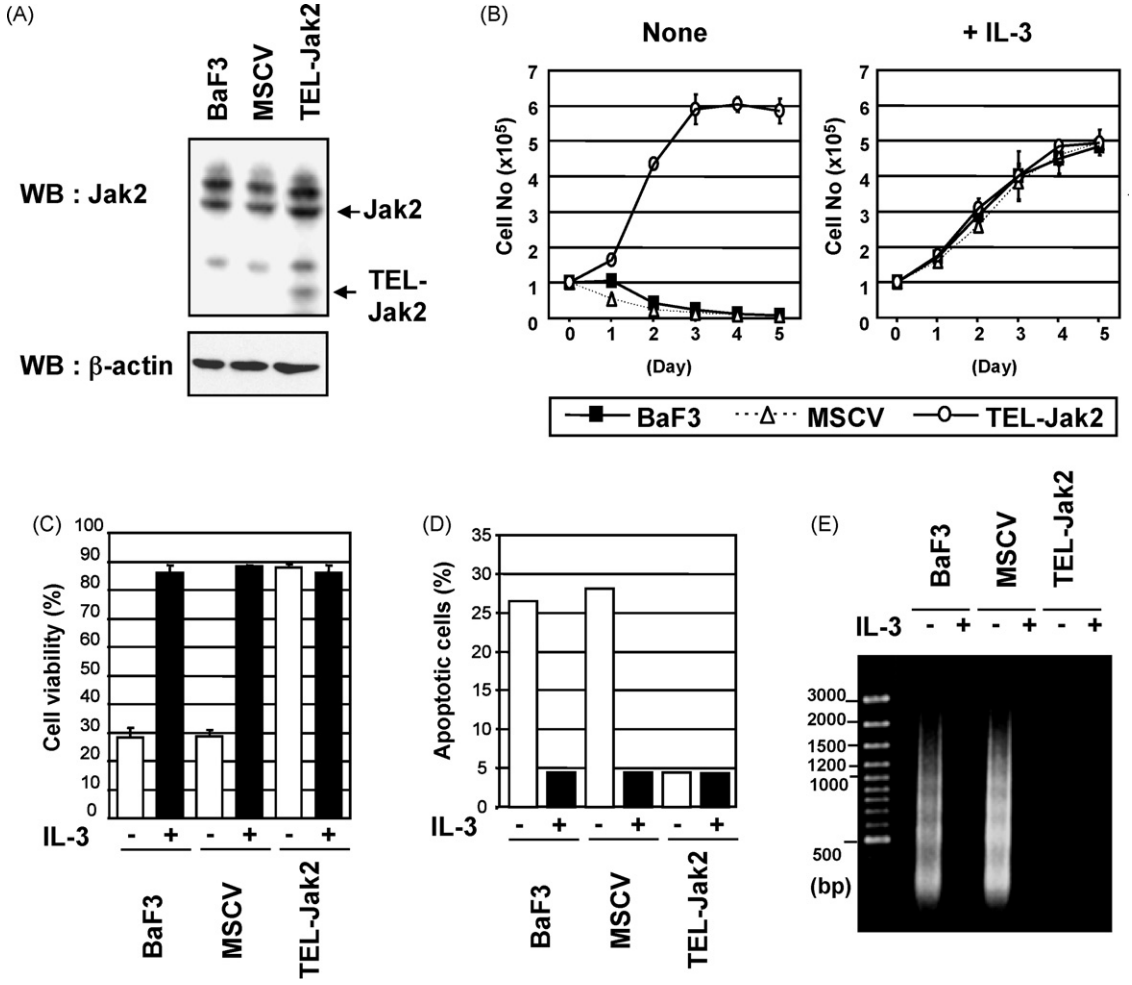


Fig. 1 – Expression of TEL-Jak2 induces cytokine-independent cell growth and inhibits apoptosis induced by IL-3 removal. (A) Expression of TEL-Jak2. Exponentially growing Ba/F3 cell lines were infected with retrovirus encoding TEL-Jak2, and then GFP-positive cells were sorted as described in Section 2. Cell lysates were immunoblotted with anti-Jak2 antibody or anti-β-actin antibody. (B) Growth of Ba/F3 cells. Parent Ba/F3 cells, empty vector-transduced Ba/F3 cells (MSCV) and TEL-Jak2-transduced Ba/F3 cells (TEL-Jak2) were washed twice with PBS and left untreated or stimulated with IL-3 (2.5 ng/ml). Surviving cells were counted using a Beckman Counter VI-Cell at the indicated times. (C) Cell viability of Ba/F3 cells. Cells were washed twice with PBS and left untreated or stimulated with IL-3 (2.5 ng/ml) for 48 h. The viability of these cells was determined by trypan blue staining. (D) Cell cycles of Ba/F3 cells. Cells were washed twice with PBS and left untreated or stimulated with IL-3 (2.5 ng/ml) for 24 h. Cells were fixed, treated with propidium iodide and subjected to flow-cytometory analysis as described in Section 2. The ratio of cells in the sub-G1 population was evaluated as apoptotic cells. (E) DNA fragmentation of Ba/F3 cells. Cells were washed twice with PBS and left untreated or stimulated with IL-3 (2.5 ng/ml) for 24 h. DNA was isolated from cells and subjected to agarose gel electrophoresis.

Interestingly, only Ba/F3/TEL-Jak2 cells could survive for 48 h after IL-3 deprivation, as shown in Fig. 1C. The viability of these cells was monitored for 5 days and Ba/F3/TEL-Jak2 cells continued to survive (data not shown). In the presence of IL-3, cell death was not detected in these three cell lines for the indicated times. We next determined different phases of cell cycle distribution in these cells following 24 h of IL-3 deprivation. In parental Ba/F3 cells and Ba/F3/MSCV cells, the percentage of cells in the S phase was reduced from 24.0% to 7.4% and from 23.4% to 8.2%, respectively (data not shown). In addition, there was an increase in the sub-G1 phase, which was consistent with apoptotic cells from 4.5% to 26.5% in Ba/F3 cells, and from 4.5% to 28.1% in Ba/F3/MSCV cells, respectively (Fig. 1D). These data demonstrate that IL-3 deprivation induces cell cycle arrest as well as apoptosis, and both events in combination are likely to contribute to the reduced cell growth of Ba/F3 cells and Ba/F3/MSCV cells, as shown in Fig. 1B. Furthermore, the ladder pattern of DNA internucleosomal fragmentation appeared in both Ba/F3 cells and Ba/F3/MSCV cells following 24 h of IL-3 deprivation, supporting that these cells underwent apoptotic cell death (Fig. 1E). Interestingly, neither significant changes in cell cycle distribution between the absence and presence of IL-3 in TEL-Jak2-transduced Ba/F3 cells were observed. Also, DNA fragmentation was not observed in Ba/F3/TEL-Jak2 cells (Fig. 1D). Interestingly, neither significant changes in cell cycle distribution nor DNA fragmentation in TEL-Jak2-transduced Ba/F3 cells were observed in the absence of IL-3. These data demonstrate that TEL-Jak2 prevents IL-3 deprivation-induced cell cycle arrest and apoptosis.

3.2. *Licochalcone A significantly induces apoptosis of TEL-Jak2-transformed Ba/F3 cells*

Licochalcone A is a major chalcone isolated from the root of *X. liquorice*, *G. inflate* Batalin (Fig. 6) [21]. We initially sought to determine whether Licochalcone A could inhibit cell growth in Ba/F3/TEL-Jak2 cells. Strikingly, we found that the treatment of Ba/F3/TEL-Jak2 cells with Licochalcone A inhibited cell growth. As shown in Fig. 2A, Licochalcone A exhibited the inhibitory effect on cell proliferation of Ba/F3/TEL-Jak2 cells at 3 μM in the absence of IL-3. On the other hand, in the presence of IL-3, same concentration of Licochalcone A failed to suppress the proliferation of Ba/F3/MSCV and Ba/F3/TEL-Jak2 cells. Licochalcone A at concentrations higher than 10 μM was required to inhibit the cell growth of these cells in the presence of IL-3 (data not shown). This result suggested one possibility that the effect of Licochalcone A might be different depending on the cell type. In other words, we speculated one possibility Licochalcone A might be more effective to the transformed cells rather than normal cells.

This result is probably because of the differences between the IL-3 signaling and TEL-Jak2-induced signaling pathways. To further scrutinize the effect of Licochalcone A on the proliferation induced by TEL-Jak2, cells were treated with different concentrations of Licochalcone A for 48 h and BrdU incorporation was examined. Treatment of Ba/F3/TEL-Jak2 cells with Licochalcone A was also found to inhibit cell proliferation in a dose-dependent manner with the half of inhibitory concentration (IC_{50}) = 0.96 μM. In the presence of IL-3, Licochalcone A inhibited the growth of Ba/F3/MSCV cells and Ba/F3/TEL-Jak2 cells with IC_{50} = 10.4 μM and 9.6 μM, respectively (data not shown). We also investigated by trypan blue staining whether Licochalcone A was able to induce cell death. As shown in Fig. 2B, the treatment of Ba/F3/TEL-Jak2 cells with 3 μM of Licochalcone A for 48 h caused potent cell death in the absence of IL-3. On the other hand, in the presence of IL-3, 10 μM of Licochalcone A was required to cause the cell death of Ba/F3/MSCV cells and Ba/F3/TEL-Jak2 cells. Next, cells were treated with DMSO or Licochalcone A and cell cycle distributions were analyzed. In the absence of IL-3, Ba/F3/MSCV cells had already undergone apoptosis and Licochalcone A had no further effect on cell cycle distributions. When Ba/F3/TEL-Jak2 cells were treated with 3 μM of Licochalcone A for 24 h, the percentage of cells in S phase reduced from 20.7% to 7.1% (data not shown). In addition, an increase of cells in sub-G1 phase (from 5.1% to 26.7%), which was consistent with apoptotic cells, was also observed (Fig. 2C). In contrast, in the presence of IL-3, 3 μM of Licochalcone A had no effect on the cell cycle distribution of Ba/F3/MSCV cells and Ba/F3/TEL-Jak2 cells. DMSO itself had no effect on cell cycle distribution in the indicated conditions. Furthermore, in the absence of IL-3, DNA fragmentation was clearly induced by 3 μM of Licochalcone A in Ba/F3/TEL-Jak2 cells, suggesting that Licochalcone A is a potent apoptosis inducer of Ba/F3/TEL-Jak2 cells (Fig. 2D). On the other hand, in the presence of IL-3, 3 μM of Licochalcone A failed to induce apoptosis of both Ba/F3/MSCV cells and Ba/F3/TEL-Jak2 cells, indicating that IL-3 protects cells from Licochalcone A-induced apoptosis.

3.3. *Licochalcone A exhibited no effect on kinase activity of TEL-Jak2*

To further investigate the inhibitory mechanism of Licochalcone A on TEL-Jak2-mediated proliferation and anti-apoptotic action, we first tested whether Licochalcone A could directly inhibit TEL-Jak2 by *in vitro* kinase assay. However, Licochalcone A had no effect on the activity of TEL-Jak2 (Fig. 3A). Since Jak2 activation is positively regulated by the phosphorylation of $Y^{1007/1008}$ within the activation loop, the effect of Licochalcone A on its tyrosine phosphorylation state at $Y^{1007/1008}$ was examined by immunoblotting analysis. TEL-Jak2 is constitutive active and tyrosine phosphorylation at $Y^{1007/1008}$ was still observed in the absence of IL-3. Consistently, after Licochalcone A treatment, the tyrosine phosphorylation level of $Y^{1007/1008}$ was not affected, suggesting that Licochalcone A did not directly inhibit TEL-Jak2 activity (Fig. 3B).

3.4. *Licochalcone A specifically inhibits Stat3 activation induced by TEL-Jak2*

Next, we examined the effect of Licochalcone A on downstream molecules of TEL-Jak2, including MAP kinases and Stats. In the Ba/F3/MSCV cells, IL-3-induced ERK, JNK, Stat3, and Stat5 activation, as shown in Fig. 4A. In the Ba/F3/MSCV cells, the constitutive activation of p38 MAPK was detected regardless of IL-3 stimulation (data not shown). Expression of TEL-Jak2-induced IL-3-independent activation of ERK, Stat3 and Stat5, except JNK. Interestingly, Licochalcone A specifically inhibited TEL-Jak2-induecd Stat3 phosphorylation,

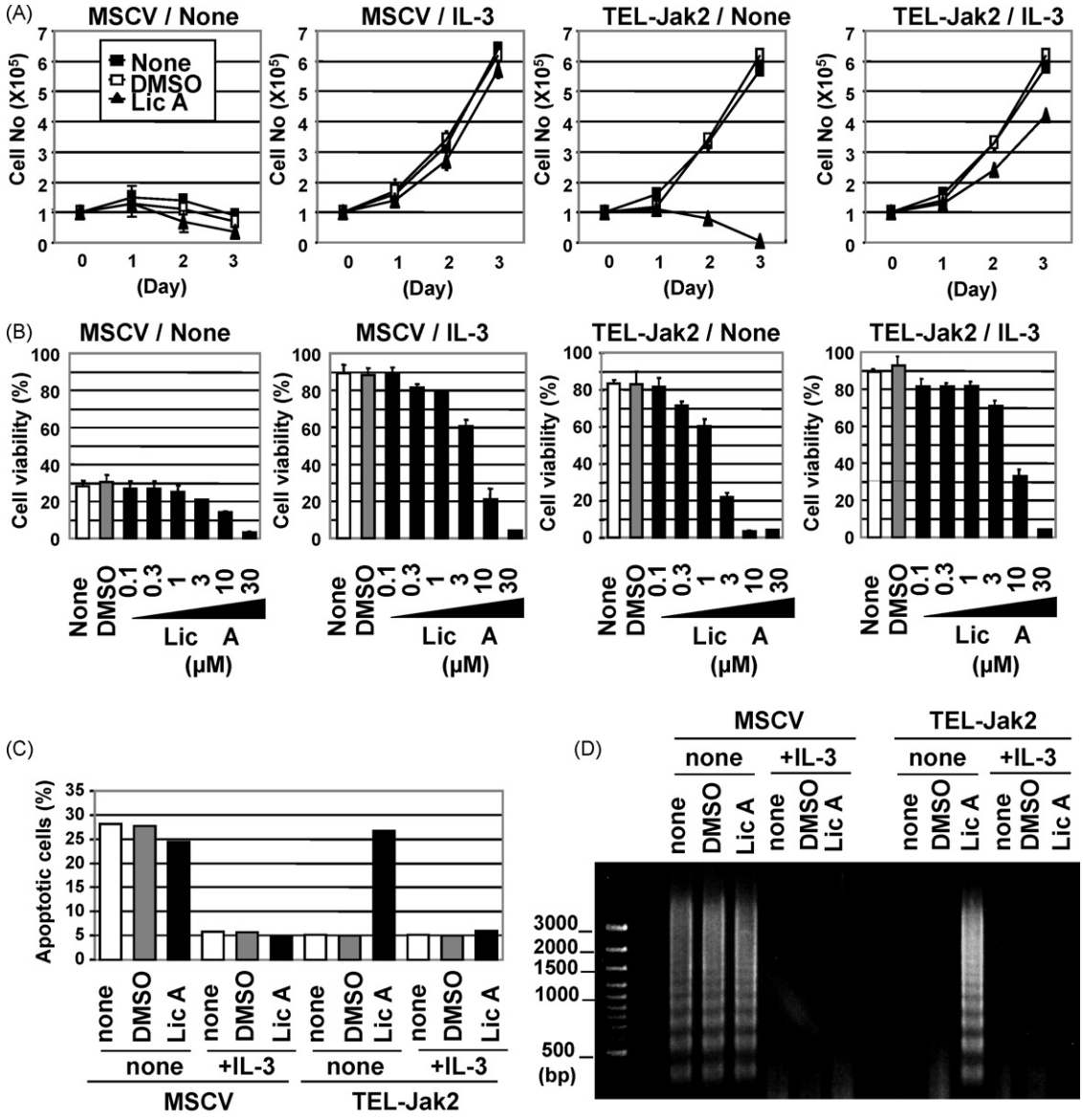


Fig. 2 – Licochalcone A significantly induced the apoptosis of TEL-Jak2-transduced Ba/F3 cells. (A) Inhibitory effect of Licochalcone A on TEL-Jak2-mediated cell growth. Empty vector-transduced Ba/F3 cells (MSCV) and TEL-Jak2-transduced Ba/F3 cells (TEL-Jak2) cultured in the absence or presence of IL-3 (2.5 ng/ml) were incubated with Licochalcone A for the indicated times. Surviving cells were counted using a Beckman Counter VI-Cell at the indicated times. (B) Inhibition of TEL-Jak2-transduced cell viability by Licochalcone A. Cells cultured in the absence or presence of IL-3 (2.5 ng/ml) were incubated with the indicated concentration of Licochalcone A for 48 h. The viability of these cells was determined by trypan blue staining. (C) Increased apoptosis of TEL-Jak2-transduced Ba/F3 cells by Licochalcone A. Cells cultured in the absence or presence of IL-3 (2.5 ng/ml) were incubated with Licochalcone A (3 μM) for 12 h. Cells were fixed, treated with propidium iodide and subjected to flow-cytometory analysis, as described in Section 2. The ratio of cells in the sub-G1 population was evaluated as apoptotic cells. (D) DNA fragmentation in TEL-Jak2-transduced Ba/F3 cells by Licochalcone A. Cells cultured in the absence or presence of IL-3 (2.5 ng/ml) were incubated with Licochalcone A (3 μM) for 16 h. DNA was isolated from cells and subjected to agarose gel electrophoresis. In the each data, Licochalcone A was indicated as Lic A.

whereas it had no effect on ERK and Stat5 activation. Also, in the presence of IL-3, Licochalcone A showed the inhibitory effect of Stat3 activation on Ba/F3/MSCV cells as well as on Ba/F3/TEL-Jak2 cells. Furthermore, the nuclear translocation of Stat3 was examined using nuclear extracts prepared after treatment with 3 μM of Licochalcone A in the absence or presence of IL-3. As shown in Fig. 4B, Licochalcone A strongly inhibited nuclear translocation of Stat3 in unstimulated Ba/F3/TEL-Jak2 cells. Also, in the presence of IL-3, Licochalcone A inhibited the nuclear translocation of Stat3 in both Ba/F3/MSCV and Ba/F3/TEL-Jak2 cells. On the other hand, Licochalcone A did not affect the translocation of Stat5 induced by both

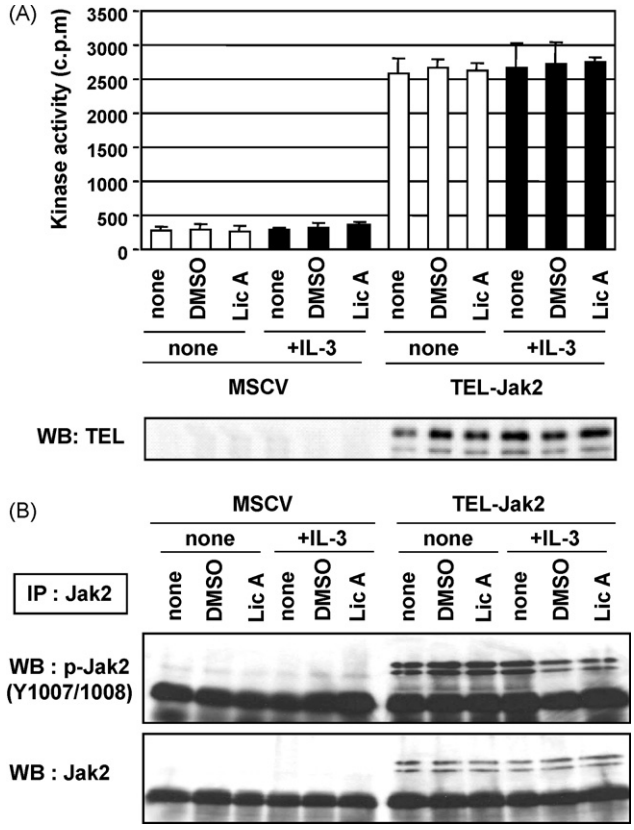


Fig. 3 – Licochalcone A exhibited no effect on kinase activity of TEL-Jak2. (A) No change in TEL-Jak2 activity by Licochalcone A. Empty vector-transduced Ba/F3 cells (MSCV) and TEL-Jak2-transduced Ba/F3 cells (TEL-Jak2) cultured in the absence or presence of IL-3 (2.5 ng/ml) were incubated with 3 μM of Licochalcone A for 6 h. (A) *In vitro* kinase activity of TEL-Jak2 was measured in the presence of a synthetic substrate peptide. Phosphorylated peptides were measured by scintillation counter (upper). Immunoprecipitated samples were separated by SDS-PAGE and blotted with anti-TEL antibody (bottom). (B) Cell lysates were subjected to immunoprecipitation using an antibody against Jak2. And then, active Jak2 and protein amount of Jak2 were analyzed by immunoblot. In the each data, Licochalcone A was indicated as Lic A.

IL-3 and the expression of TEL-Jak2, suggesting that Licochalcone A had the ability to specifically inhibit Stat3.

3.5. *Stat3 is an essential molecule for TEL-Jak2-induced oncogenesis*

In order to investigate how Stat3 activation contributes to TEL-Jak2-induced transformation, siRNA against Stat3 was employed. Transfection of Stat3 siRNA effectively decreased Stat3 expression by greater than 80%, as compared to transfection with control siRNA both in Ba/F3/MSCV cells and Ba/F3/TEL-Jak2 cells (Fig. 5A). Interestingly, Stat3 siRNA significantly inhibited IL-3-independent survival of Ba/F3/TEL-Jak2 cells (Fig. 5B). In addition, it was found that Stat3 siRNA significantly increased apoptotic cell death of the Ba/F3/TEL-Jak2 cells analyzed by sub-G1 population (Fig. 5C) and DNA ladder (Fig. 5D) in absence of IL-3. However, in the presence of IL-3, the transfection of Stat3 siRNA had little effect on the viability and cell cycle patterns of both Ba/F3/MSCV and Ba/F3/TEL-Jak2 cells, suggesting that knockdown of Stat3 was not able to induce apoptosis in these cells when IL-3 signaling was activated. Thus, these data indicate that Stat3 is specifically an indispensable molecule for cell proliferation and the anti-apoptotic effect induced by TEL-Jak2. In contrast, it is presumed that Stat3 is not essential for IL-3-induced survival of Ba/F3/MSCV and Ba/F3/TEL-Jak2 cells.

3.6. *4- and 4′-OH group in Licochalcone A is important for its inhibitory effect on Stat3*

We next investigated the structural requirement of Licochalcone A in TEL-Jak2-transformed cells. As shown in Fig. 6, Licochalcone A belongs to the chalcones, which are characterized by being α,β-unsaturated biphenyl ketones. In Licochalcone A, 4-(R1)- and 4′-(R2)-hydroxyl groups (–OH) were substituted with four different groups: methoxyl groups (–OCH_3), acetoxy groups (–$OCOCH_3$), carboxymethoxyl groups (–OCH_2COOH) and glutaric acid groups (–OCO $(CH_2)_3COOH$), as illustrated in Fig. 6. Among four kinds of synthetic derivatives, only compound 3 was unable to inhibit cytokine-independent cell growth by TEL-Jak2, whereas the other compounds inhibited cell growth induced by TEL-Jak2 to the same extent as Licochalcone A (Fig. 7A). In addition, as a result of BrdU incorporation analysis, compound 3 had no effect on the proliferation of the Ba/F3/TEL-Jak2 cells. On the other hand, compounds 1, 2 and 4 effectively inhibited the proliferation of Ba/F3/TEL-Jak2 cells with IC_{50} = 1.2 μM (compound 1), IC_{50} = 0.25 μM (compound 2) or IC_{50} = 2.1 μM (compound 3), respectively (data not shown). To further investigate the effects of Licochalcone A derivatives, cell viabilities were examined by trypan blue staining after treatment of these derivatives. Compounds 1, 2 and 4 significantly reduced the viability of Ba/F3/TEL-Jak2 cells in a dose-dependent manner (Fig. 7B). These compounds were able to induce cell death completely at a concentration of 10 μM as well as Licochalcone A. On the other hand, compound 3 did not induce cell death at the high concentration of 30 μM during 3-day incubation. The Ba/F3/TEL-Jak2 cells were treated with 3 μM of either compound 1, 2 or 4 for 24 h, the percentage of cells in S phase reduced from about 23.7% to about 7% (data not shown), and the percentage of cells in sub-G1 phase increased from 5.3% to about 26% (Fig. 7C). Furthermore, after treatment with 3 μM of compounds 1, 2 and 4 for 24 h, DNA fragmentation was clearly observed, suggesting that these Licochalcone A derivatives are potent apoptosis inducers for the Ba/F3/TEL-Jak2 cells (Fig. 7D). In contrast, compound 3 had no effect on cell cycle distribution in Ba/F3/TEL-Jak2 cells and did not induce DNA fragmentation. Interestingly, other compounds significantly inhibited the activation of Stat3 but not Stat5, whereas only compound 3 had no effect of Stat3 activation induced by TEL-Jak2 (Fig. 7E). Thus, we assumed that 4-and 4′-OR groups in Licochalcone A are critical for its inhibitory effect on TEL-Jak2-induced transformation. In addition, since the compound in which 4-(R1)-hydroxyl groups (–OH) was substituted with hydrogen group (–H), still has an inhibitory effect on TEL-Jak2-induced Stat3 activation, it is considered that

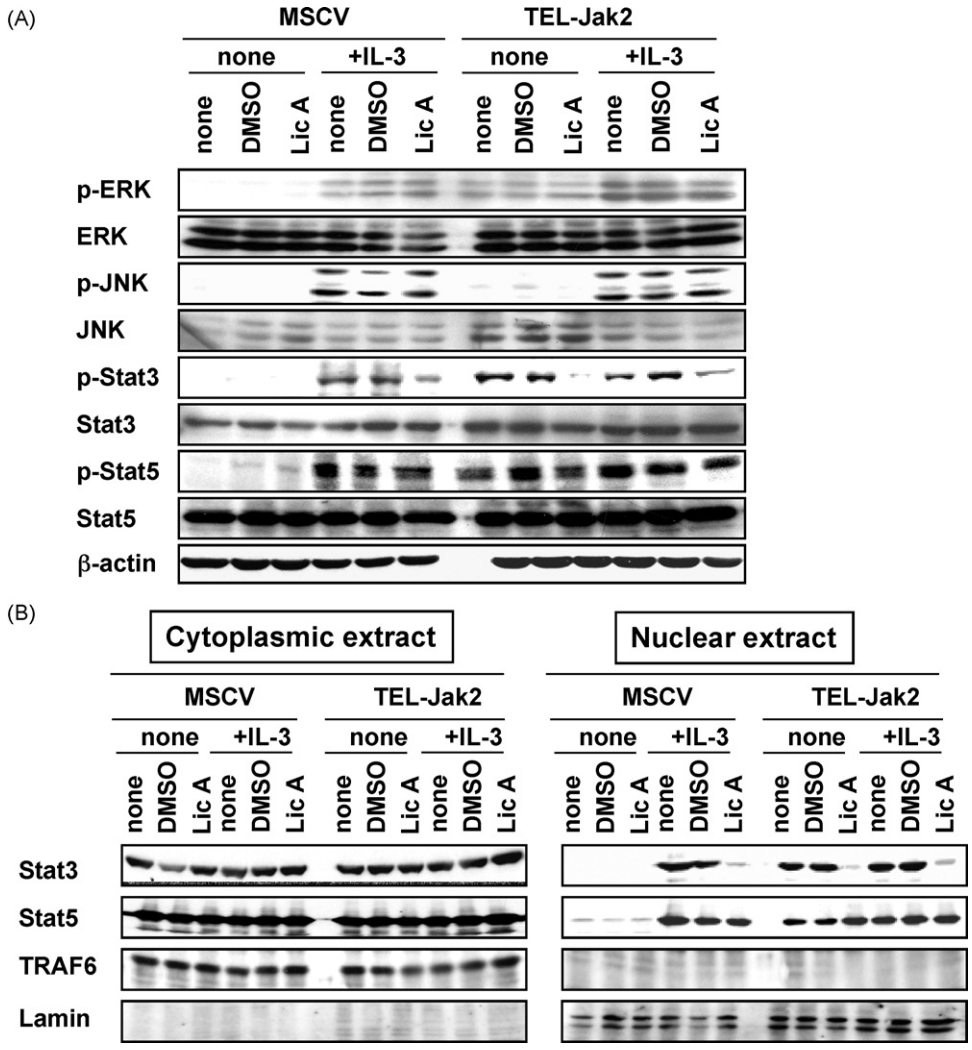


Fig. 4 – Licochalcone A specifically inhibited Stat3 activation induced by TEL-Jak2. (A) Specific inhibition of Stat3 activation by Licochalcone A. Cells cultured in the absence or presence of IL-3 (2.5 ng/ml) were incubated with 3 μM of Licochalcone A for 6 h. Cell lysates were immunoblotted with anti-phospho-ERK antibody, anti-ERK antibody, anti-phospho-JNK antibody, anti-JNK antibody, anti-phospho-Stat3 antibody, anti-Stat3 antibody, anti-phospho-Stat5 antibody, anti-Stat5 antibody or anti-β-actin antibody. (B) Inhibition of Stat3 nuclear localization by Licochalcone A. Cells cultured in the absence or presence of IL-3 (2.5 ng/ml) were incubated with 3 μM of Licochalcone A for 6 h. Cytoplasmic extracts and nuclear extracts were prepared and analyzed by immunoblot with anti-Stat3, anti-Stat5, anti-TRAF6, or anti-lamin antibodies, respectively. In this figure, Licochalcone A was indicated as Lic A.

two hydroxyl groups in Licochalcone A are not necessary for its inhibitory effect (data not shown).

4. Discussion

Our results strongly suggest that Licochalcone A specifically inhibits Stat3 activation, thereby potently inducing apoptosis of TEL-Jak2-transformed cells. Stat3 is a latent cytoplasmic transcription factor that performs a variety of functions in regulating cell growth, inflammation [26] and early embryonic development [27]. Interestingly, Stat3 is frequently constitutively activated in cancer cells, such as breast carcinoma and leukemia [28,29]. Furthermore, the expression of a constitutively active dimmer of Stat3 transforms cultured cells and promotes tumor formation in nude mice [30] and thus, Stat3 is classified as an proto-oncogene. It is also recognized that Stat3 plays pivotal roles in malignant transformation by increasing the expression of various genes such as matrix metalloproteinase 2 (MMP2), matrix metalloproteinase 9 (MMP9) [31] and vascular endothelial growth factor (VEGF) [32,33], which play important roles in cell growth, tumorgenesis and angiogenesis. Recently, DNA-methylation of the promoter region of suppressors of cytokine signaling 1 (SOCS1) and SOCS3 has been identified in the tumorgenesis of liver cancer, lung cancer, carcinoma of the head and neck (HNSCC) and hepatocellular carcinoma (HCC), suggesting that the expression of the SOCS family can be silenced in malignant tumor [34,35]. As a result of the loss of SOCS1 or SOCS3 functions in these cancer cells, Stat3 is considered to deviate from a precise regulatory mechanism,

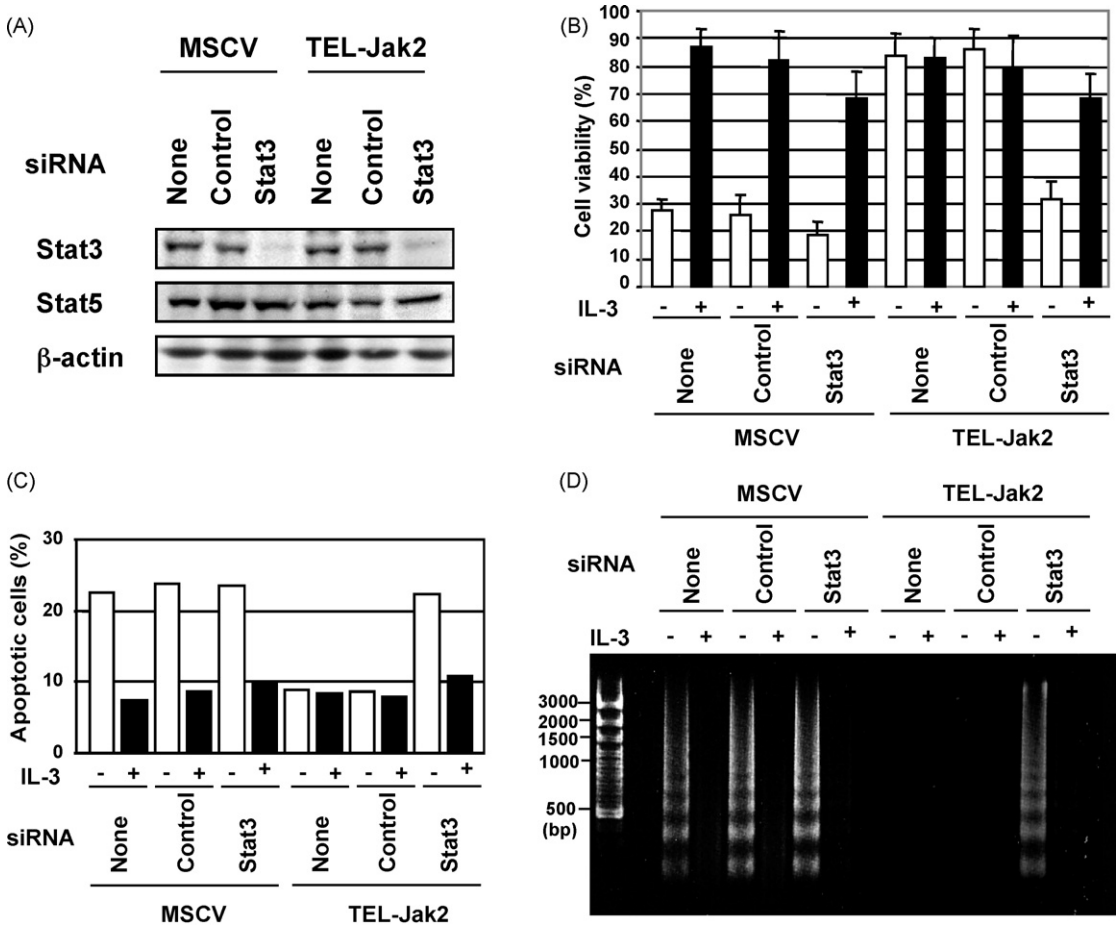
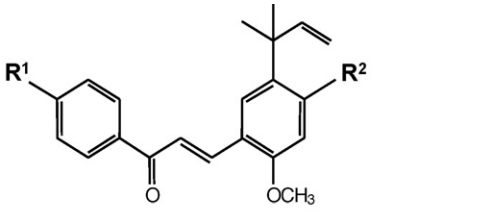


Fig. 5 – Stat3 is essential for TEL-Jak2-induced transformation. (A) Knock down of Stat3 by siRNA. Empty vector-transduced Ba/F3 cells (MSCV) and TEL-Jak2-transduced Ba/F3 cells (TEL-Jak2) were transfected with siRNA to Stat3 as described in Section 2. Cell lysates were immunoblotted with anti-Stat3 antibody, anti-Stat5 antibody or anti-β-actin antibody. (B) Reduced cell viability of TEL-Jak2-transduced cells by siRNA to Stat3. After transfection of siRNA to Stat3, cells were cultured in the absence or presence of IL-3 (2.5 ng/ml) and incubated for 24 h. The viability of these cells was determined by trypan blue staining. (C) Increased apoptosis of TEL-Jak2-transduced cells by siRNA to Stat3. After transfection of siRNA to Stat3, cells were cultured in the absence or presence of IL-3 (2.5 ng/ml) for 24 h. Cells were fixed, treated with propidium iodide and subjected to flow-cytometory analysis as described in Section 2. The ratio of cells in the sub-G1 population was evaluated as apoptotic cells. (D) Increased DNA ladder of TEL-Jak2-transduced cells by siRNA to Stat3. After transfection of siRNA to Stat3, cells were cultured in the absence or presence of IL-3 (2.5 ng/ml) for 24 h. DNA was isolated from cells and subjected to agarose gel electrophoresis.



Licochalcone A	R^1, R^2=OH
Compound 1	R^1, R^2=OCH_3
Compound 2	R^1, R^2=$OCOCH_3$
Compound 3	R^1, R^2=OCH_2COOH
Compound 4	R^1, R^2=$OCO(CH_2)_3COOH$

Fig. 6 – Derivatives of Licochalcone A. All of the compounds were synthesized as described in Section 2.

leading to the up-regulation of cell proliferation and tumor development [36,37]. By considering a number of observations, Stat3 is thought to be a suitable target for anti-cancer drugs.

Several previous studies have reported that the expression of TEL-Jak2 also induced constitutive activation of Stat3 as well as Stat5 [11,12], however, no direct evidence has shown that constitutive activation of Stat3 could confer the advantage of cell growth. So far, Schwaller et al. [25] have demonstrated that Stat5 is necessary for the myelo- and lympho-proliferative disease induced by TEL-Jak2 using a genetic approach. While the mice transplanted with bone marrow expressing TEL-Jak2 displayed acute fatal myelo- and lympho-proliferative syndrome, the reconstitution of Stat5-deficient bone marrow failed to exhibit the severe hematopoietic disorders [25]. In this study, we demonstrated that Stat3 is also essential for

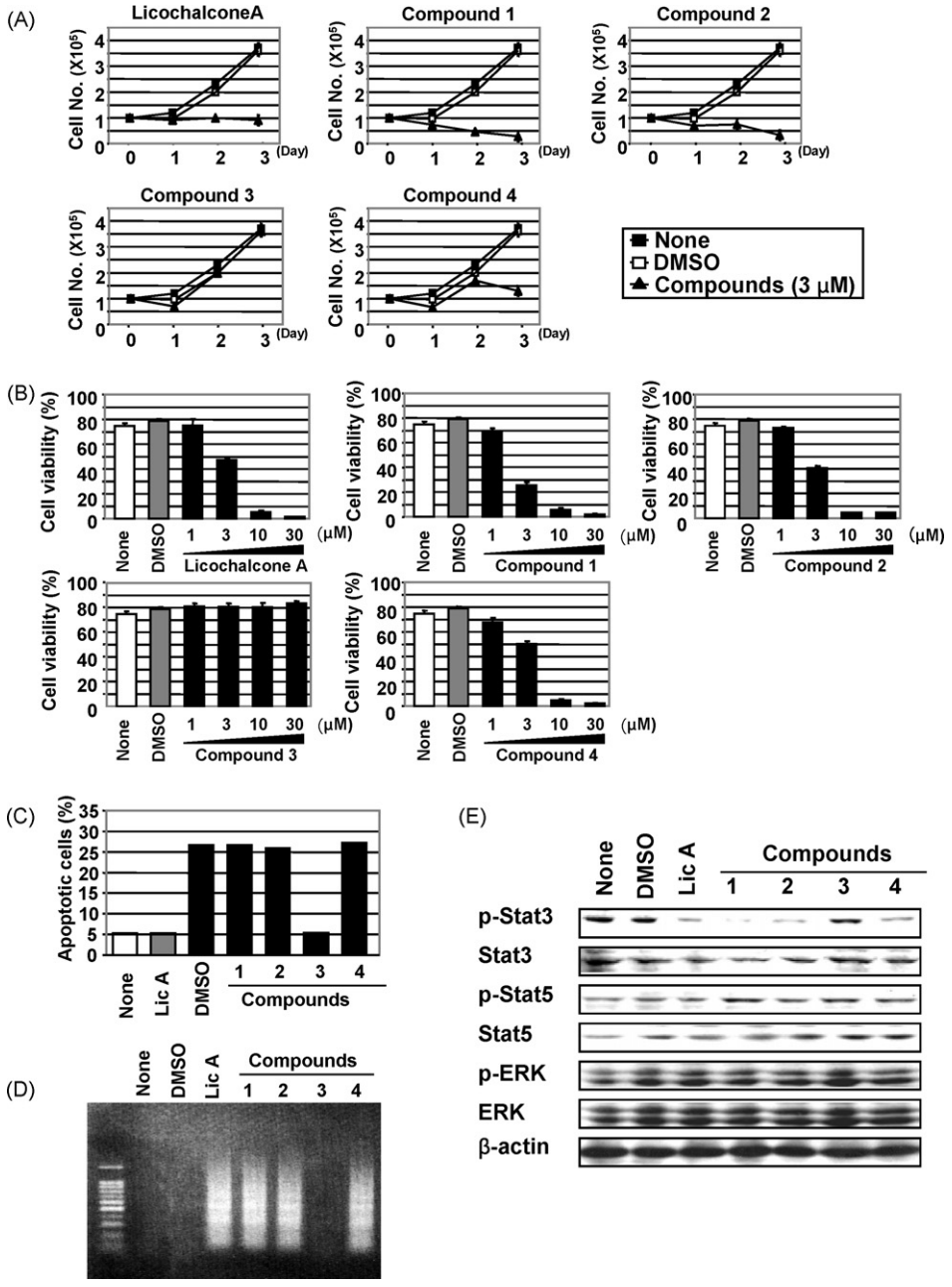


Fig. 7 – A Licochalcone A-derived compound failed to inhibit Stat3 activation and to induce the apoptosis of TEL-Jak2-transduced Ba/F3 cells. (A) Inhibitory effect of Licochalcone A derivatives on cell growth induced by TEL-Jak2. TEL-Jak2-transduced Ba/F3 cells were incubated with Licochalcone A derivatives for the indicated times. Surviving cells were counted using a Beckman Counter VI-Cell at the indicated times. (B) Inhibition of TEL-Jak2-transduced cell viability by Licochalcone A derivatives. TEL-Jak2-transduced Ba/F3 cells were incubated with Licochalcone A derivatives for 48 h. The viability of these cells was determined by trypan blue staining. (C) Increased apoptosis in TEL-Jak2-transduced Ba/F3 cells by Licochalcone A derivatives. TEL-Jak2-transduced Ba/F3 cells were incubated with Licochalcone A derivatives (3 μM) for 12 h. Cells were fixed, treated with propidium iodide and subjected to flow-cytometory analysis as described in Section 2. The ratio of cells in the sub-G1 population was evaluated as apoptotic cells. (D) Induction of DNA fragmentation in TEL-Jak2-tranduced BaF3 cells by Licochalcone A derivatives. TEL-Jak2-transduced Ba/F3 cells were incubated with Licochalcone A derivatives for 16 h. DNA was isolated from cells and subjected to agarose gel electrophoresis. (E) Inhibition of TEL-Jak2-induced Stat3 activation by Licochalcone A derivatives. TEL-Jak2-transduced Ba/F3 cells were incubated with Licochalcone A derivatives (3 μM) for 16 h. Cell lysates were immunoblotted with anti-phospho-Stat3, anti-Stat3, anti-phospho-Stat5, anti-Stat5, anti-phospho-ERK or anti-ERK antibodies, respectively.

TEL-Jak2-mediated oncogenesis, as shown in Fig. 5. The knockdown of Stat3 using siRNA-induced apoptosis of TEL-Jak2-transformed Ba/F3 cells (Ba/F3/TEL-Jak2 cells) in the absence of IL-3, suggesting that Stat3 is indeed important for TEL-Jak2-induced transformation. Interestingly, in the presence of IL-3, drastic apoptosis was not induced by knockdown of Stat3 in both empty vector-transduced cells and TEL-Jak2-transduced cells. Compared to the contribution of Stat3 to TEL-Jak2-mediated cell survival, most unlikely in the IL-3 signaling pathway, Stat3 seems to be essential for cell growth and survival. Furthermore, as shown in Fig. 3, whereas the activation of JNK was clearly induced by IL-3, the expression of TEL-Jak2 did not induce JNK activation in our system. Although the role of JNK activation in cell growth and survival is still unclear, these results suggest that the TEL-Jak2-induced signaling pathway is not necessarily the same as the IL-3-signaling pathway. Moreover, this idea seems to be one reason why the contribution of Stat3 is different in TEL-Jak2-induced signaling and IL-3 signaling. However, the previous study reported that the expression of TEL-Jak2 in Ba/F3 cells results in constitutive activation of JNK activity determined by *in vitro* kinase assay [13]. Whereas, they used a transfection method to obtain stable transfectant expressing TEL-Jak2, we established Ba/F3 cells expressing TEL-Jak2 (Ba/F3/TEL-Jak2 cells) by retrovirus infection. It can be imagined that the expression level of TEL-Jak2 in our Ba/F3 cells was much lower than in their cell system, suggesting that one possible reason for the difference in JNK activation could be the different expression level of TEL-Jak2. Because in our experiments the expression level of TEL-Jak2 was almost the same as endogenous Jak2, shown in Fig. 1A, it can be expected that our expression system will reflect the actual expression level of TEL-Jak2 in leukemia patients.

In this study, we clarified that Stat3 plays a critical role in the transformation induced by TEL-Jak2, and found that Licochalcone A specifically inhibited its activation, leading to apoptosis of TEL-Jak2-transduced cells.

Licochalcone A is 5-(1,1-dimethyl-2-propenyl)-4, 4′-dihydroxy-2-methoxychalcone, shown in Fig. 6. To understand the precise mechanism of Licochalcone A by examining the correlation of the structure and its activity, we used Licochalcone A derivatives, in which 4 and 4′-OH were substituted with four different groups. Interestingly, even when TEL-Jak2-transformed cells were incubated with 30 μM of compound 3, the cells survived, suggesting that the substitution of –OH with $–OCH_2COOH$ failed to induce apoptosis of TEL-Jak2-transduced cells. To consider why only compound 3 failed to induce apoptosis, log *P* of Licochalcone A derivatives was calculated by the Spartan '04. Log *P* is an index showing the hydrophobility of chemical compounds and the value of calculated log *P* of each compound was as follows: Licochalcone A, 4.71; compound 1, 5.23; compound 2, 4.66; compound 3, 3.76 and compound 4, 4.83. Since lipophilicity of compound 3 is the lowest among these, it is easily speculated that compound 3 had difficulty to penetrate into the cell to exhibit its activity because of its low hydrophobility.

Licochalcone A is isolated from licorice roots, which are known to contain several other kinds of chalcones, such as Licochalcone B, Licochalcone C, Licochalcone D, Echinatin, and Isoliquiritigenin. All compounds have a common 4,4′-dihydroxy chalcone structure, and we found that all these compounds inhibited the survival of TEL-Jak2-transformed cells, although their detailed mechanism has not been analyzed (data not shown). Licorice roots have been used as traditional medicine all over the world for years, and are also recognized as a safe medicine with few side effects. Taken together, our investigation that extracts from licorice roots have anti-tumorgenesis activity is expected to bring about the possible development of Licochalcone derivatives as a powerful and safe anti-cancer drug.

Several tyrosine kinases, such as Src and EGF-receptor, have also been reported to activate Stat3 [38,39], leading to stimulate the malignant transformation of cultured cells [40,41]. Because Stat3 represents a point of convergence for many oncogenic as well as angiogenic events, Stat3 is a promising molecular target for powerful intervention in cancer therapy. Thus, Licochalcone A could be expected to inhibit not only the TEL-Jak2-induced signaling pathway but also multiple angiogenic signaling pathways.

In our current research, Licochalcone A induces programmed cell death of both of wild type and TEL-Jak2-expressing Ba/F3 cells. On the other hand, the experiment using Stat3 RNAi told us a striking results, Stat3 likely seems to be essential for cell survive of TEL-Jak2-expressing Ba/F3 cells but not for wild type. The discrepancy between the experiments using Licochalcone A and Stat3 RNAi could be occurred, since Licochalcone A may target not only Stat3 but also other important signaling molecules.

The detailed mechanism, by which Licochalcone A inhibits Stat3 activation, is still unclear. According to our current results, Licochalcone A has to target Stat3 or its interacting proteins but not TEL-Jak2. Licochalcone A might abolish the physical interaction between TEL-Jak2 and Stat3 and cancel the phosphorylation of Stat3 by TEL-Jak2. It may be necessary to test whether Licochalcone A binds to Stat3 or its protein complex in the future experiments. Also, several derived compounds of Licochalcone A probably inhibit Stat3 activation through similar mechanism. The relationship between structural property of side-chain of derivatives and their interaction with Stat3 will have to be elucidated.

In our study, the derivatives from Licochalcone A showed various effective concentrations to TEL-Jak2-expressing cells. This may provide us a clue to develop the more specific derivative by modifying the basic backbone of Licochalcone A.

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