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Running head: EFFECTS OF LITHIUM ON INSECT CELLS

Effects of lithium on the secretory production of recombinant antibody from insect cells

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Abstract

Monoclonal antibodies and antibody fragments are widely used in therapeutics and diagnoses. While mammalian cells serve as the host cells for antibody production, insect cells can produce large quantities of secretory antibodies in serum-free suspension cultures. The effects of lithium on the processes of autophagy and apoptosis in mammalian cells are well chronicled. In the present study, stably transformed insect cells, which produce an engineered antibody molecule, were cultured with lithium chloride in a serum-free medium. Treatment with lithium chloride induced autophagy and apoptosis in recombinant insect cells and led to increases in the yields of secreted antibodies.

Key words Insect cell culture · Recombinant protein production · Lithium chloride · High Five cells · Antibody

1 **Introduction**

2
3 In recent years diverse technologies have been developed for the efficient production of
4 engineered antibody molecules for use in therapeutics and diagnoses. Mammalian CHO
5 and 293T cells are the primary producers of recombinant antibodies (Kim et al. 2012;
6 Mohan et al. 2008; Omasa et al. 2010), whereas *Escherichia coli* is only used in partial
7 applications (Katsuda et al. 2012; Menzel et al. 2008; Puttikhunt et al. 2008; Schlapschy
8 and Skerra 2011). Insect cells offer several advantages for the secretory production of
9 recombinant antibody molecules (Furuta et al. 2012; Palmberger et al. 2011; Yamaji et al.
10 2008). First, by comparison with *E. coli*, the processes such as post-translational
11 processing and modifications and protein folding that occur in insect cells are almost
12 similar to those processes in mammalian cells. Second, the rate of protein production in
13 insect cells is generally higher than that in mammalian cells. Third, it is easier to achieve
14 a serum-free suspension culture with high cell density when using insect cells compared
15 with using mammalian cells. Therefore, insect cells can be used to produce large amounts
16 of recombinant proteins with complicated structures such as antibodies.

17 Lithium is currently used as a medicine for neuronal diseases such as bipolar disorder,
18 because it promotes cell proliferation and inhibits cell death (Dell’Osso et al. 2016;
19 Forlenza et al. 2014; Sarkar and Rubinsztein 2008). One of the functions of lithium in cell
20 proliferation and inhibition of cell death is the enhancement of autophagy (Motoi et al.
21 2014). Recent reports have shown that autophagy plays an important role in the secretory
22 production of antibodies in CHO cells and plasma cells (Lee and Lee 2012; Pengo et al.
23 2013). Another function of lithium in both cell proliferation and the inhibition of cell
24 death is related to apoptosis, and it is the cell type that determines whether lithium will
25 attenuate apoptosis or induce it. For example, lithium attenuates the apoptosis of PC12

cells (Zeng et al. 2016), but it promotes the apoptosis of some cancer cells (Adler et al. 2010; Kunnimalaiyaan et al. 2007; Li et al. 2015; Peng et al. 2013; Wang et al. 2008; Yin et al. 2013).

To date, there has been no reports of the effects that lithium exerts on recombinant protein production in insect cells. In the present study, we examined the effects of lithium on insect cells that secrete an engineered antibody molecule in a serum-free culture. Addition of lithium promoted recombinant antibody production in insect cells, probably due to the induction of autophagy. Treatment of insect cells with lithium may allow efficient production of recombinant proteins.

Materials and methods

Insect cell line, medium, and supplements

Stably transformed 1G3 cells that produce a single-chain variable fragment fused with the Fc region of human IgG1 (scFv-Fc) were previously established from *Trihoplusia ni* BTI-TN-5B1-4 (High Five) insect cells (Sonoda et al. 2012) and used in the present study. The cells were maintained at 27 °C in a serum-free medium Express Five SFM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 2.41 g/L L-glutamine (Thermo Fisher Scientific), 0.8 g/L G418 (Nacalai tesque, Kyoto, Japan), and 10 mg/L gentamycin sulfate (Thermo Fisher Scientific). Cell density was determined by microscopically counting the number of cells with a hemocytometer. Cell viability was determined by trypan blue dye exclusion.

Lithium chloride (LiCl) and bafilomycin A1

Anhydrous LiCl (code: 20624) was purchased from Nacalai tesque, and bafilomycin A1 was from Sigma-Aldrich (St. Louis, MO, USA). LiCl was dissolved in phosphate-buffered saline (PBS), and was added to 1G3 cells. The ratio of the volume of the LiCl solution added was adjusted to equal 10% of the culture medium volume. Bafilomycin A1 was dissolved at 100 nM in dimethyl sulfoxide (DMSO), and was added to the cells. The ratio of the volume of the added bafilomycin A1 solution was adjusted to equal 1% of the medium volume.

Static culture

Cells were inoculated into 6-well cell culture plates with 2 ml of fresh medium at a cell density of 2×10^5 cells/cm³ and were statically cultivated at 27 °C. All the cells and supernatant from one of the wells served as a sample for each procedure. Each culture condition was tested over twice, and a reproducible trend was observed on repeated runs.

Suspension culture

Cells were suspended in fresh medium at a cell density of 2×10^5 cells/cm³, and 20 ml of the cell suspension was transferred into a 100-ml Erlenmeyer flask. The cells were cultivated at 27 °C on a rotary shaker (90 rpm). An aliquot of the cell suspension was sampled for each procedure. Each culture condition was tested over twice, and a reproducible trend was observed on repeated runs.

Cell staining using a Cyto-ID Autophagy detection kit

Autophagosomes and nuclei in cells were stained using a Cyto-ID Autophagy detection kit (Enzo Life Sciences, Farmingdale, NY, USA) following the manufacturer's protocol. Cells were inoculated with LiCl into glass-based dishes (Matsunami Glass Ind.,

Kishiwada, Japan). Bafilomycin A1 was added to the cells on day 1 after inoculation. The cells were stained using the Cyto-ID Autophagy detection kit on day 3 after inoculation and observed via the EVOS FL cell imaging system (Thermo Fisher Scientific).

Western blotting

Culture supernatants were analyzed by western blotting to evaluate the quantity of secreted scFv-Fc. Culture supernatants were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. Immunoreactive proteins were detected with alkaline phosphatase conjugated goat anti-human IgG-Fc fragment antibody (Bethyl Laboratories, Boston, MA, USA), as previously described (Ohmuro-Matsuyama et al. 2017). Relative quantities of scFv-Fc were calculated from the detected bands using ImageJ software (<http://imagej.nih.gov/ij/>; Schneider et al. 2012).

Enzyme-linked immunosorbent assay (ELISA)

Culture supernatants were also analyzed by ELISA to evaluate the antigen-binding activity of scFv-Fc using peroxidase conjugated goat anti-human IgG-Fc fragment antibody (Bethyl Laboratories), as previously described (Furuta et al. 2012).

Results

Antibody production in a static culture with LiCl

Stably transformed 1G3 cells that produce scFv-Fc were inoculated in a serum-free medium with LiCl at concentrations of 3.3, 10, and 30 mM (day 0), and were statically cultivated. The treatments with 3.3 and 10 mM LiCl promoted cell growth during days

1–6 (Fig. 1A), and all the LiCl treatments displayed enhanced viability on days 6–10 (Fig. 1B). Higher concentrations of LiCl (90 and 270 mM), however, significantly inhibited cell growth (data not shown). The antigen-binding activity in the culture supernatant was determined via ELISA. The binding activities increased with LiCl concentration, and the increases by the additions of 10 and 30 mM LiCl were remarkable after day 6 (Fig. 1C). Culture supernatants were also analyzed by western blotting to evaluate the quantity of secreted scFv-Fc, and relative quantities of scFv-Fc were calculated from the detected bands. The amounts of scFv-Fc detected by western blot analysis almost corresponded with the ELISA data (Fig. S1).

Subsequently, LiCl was added to 1G3 cells on day 4 after inoculation, since the effect of LiCl on scFv-Fc production was significant on and after day 6. Higher cell densities were observed on day 6 with all the LiCl treatments, and higher cell viabilities were obtained with treatments with 3.3 and 10 mM LiCl during days 8–10 (Figs. 2A, B). The antigen-binding activity in the culture supernatant, however, remained almost the same in both the presence and absence of LiCl throughout the culture (Fig. 2C).

Effects of LiCl on autophagy and cell death

Above-mentioned results prompted a few questions. The first question is why LiCl treatment promoted the proliferation of insect cells in the growth phase and improved viability in the declining phase. Another question is whether or not LiCl induced autophagy and inhibited apoptosis in insect cells. To investigate these questions, we observed autophagosomes (Fig. 3A) as well as the shapes of cells and nuclei (Fig. 3B).

Treatment with 30 mM LiCl increased both the number of cells containing autophagosomes and the fluorescent intensity of stained autophagosomes, by comparison with cells without LiCl (Fig. 3A). When 1 nM of bafilomycin A1, an autophagy inhibitor,

was added to cells treated with 30 mM LiCl on day 1 after inoculation, no autophagosome activity was observed in the cells. These observations suggest that LiCl promotes autophagy in 1G3 cells.

Damaged cells were increased by LiCl treatment, and some cells treated with 10 mM LiCl showed blebbing of the cell membrane (apoptosis body) and a fragmented nucleus, which are features of apoptotic cells (Fig. 3B). These results indicate that LiCl might promote the apoptotic death of insect cells as well as autophagy.

Antibody production in a suspension culture with LiCl

Insect cells have an advantage whereby a high cell density is easily achieved in a serum-free suspension culture. Finally, we examined the effects of LiCl on 1G3 cells in a shake-flask culture. A low concentration (3.3 mM) of LiCl enhanced the cell proliferation during days 3–6 (Fig. 4A). The viability of cells during days 6–12 improved with LiCl concentration (Fig. 4B). The concentration of scFv-Fc with antigen-binding activity was increased by the LiCl treatment after day 6, and the scFv-Fc yield in the presence of LiCl reached more than 2-fold during days 8–12 compared with the absence of LiCl (Fig. 4C). The quantity of scFv-Fc was also increased by the LiCl treatment (Fig. S3). ◀ Fig. 4

Discussion

In the present study, the effects of LiCl on recombinant 1G3 insect cells that secrete scFv-Fc were investigated in a serum-free culture. When LiCl was added on day 0 in a static culture, the results show that an appropriate concentration of LiCl (3.3 and 10 mM) enhanced cell proliferation during the cell growth phase (Fig. 1A), and LiCl supported a relatively high viability of cells in the declining phase (Fig. 1B). The resultant increases

1 in the number of cells throughout the culture may have led to the increased yields of scFv-
2 Fc. In contrast, the treatment with 30 mM LiCl did not markedly affect the number of
3 cells compared with treatments of 3.3 and 10 mM LiCl, but the highest concentration of
4 scFv-Fc was achieved with 30 mM LiCl (Fig. 1C). This result suggests that LiCl could
5 have increased the specific productivity of 1G3 cells. When LiCl was added to 1G3 cells
6 on day 4 after inoculation, scFv-Fc production remained almost the same both with and
7 without LiCl throughout the culture (Fig. 2C). This result shows that in order to enhance
8 the scFv-Fc production in 1G3 cells LiCl should be added during the initial growth phase
9 but not in the latter periods of cell growth.

10 Microscopic observations of 1G3 cells treated with LiCl and bafilomycin A1 (Fig.
11 3A) suggest that LiCl promotes autophagy in 1G3 cells. Autophagy may inhibit cell death
12 and then increase the viability in the declining phase, which might lead to a higher level
13 of scFv-Fc production (Figs. 1B, C). Detections of apoptotic features in cells treated with
14 LiCl (Fig. 3B) might suggest the possibility that cells were selected via apoptosis caused
15 by LiCl in the initial growth phase, and that active proliferation of surviving cells led to
16 higher levels of both cell density and scFv-Fc production (Figs. 1A, C).

17 In a shake-flask culture where LiCl was added on day 0, a high cell density was
18 attained, and the yield of scFv-Fc was remarkably increased by the LiCl treatment
19 compared with the absence of LiCl (Figs. 4A, C). In at least one report, LiCl did not
20 enhance antibody production in CHO cells, but rapamycin promoted both autophagy and
21 antibody production in CHO cells (Lee and Lee 2012). Rapamycin enhances the mTOR
22 dependent pathway for autophagy. On the other hand, LiCl inhibits the mTOR dependent
23 pathway, and induces autophagy via the GSK3 β and IMPase pathways (Motoi et al. 2014).
24 Via these pathways induced by LiCl protein production could be stimulated in insect cells,
25 although these pathways might not be important for antibody production in CHO cells.

Conclusion

Treatment with LiCl may promote autophagy and apoptosis in insect cells. After the latter period of cell growth LiCl promotes recombinant protein production, probably due to the induction of autophagy. Treatment of insect cells with LiCl is an easy, economical, and useful method to promote protein production.

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Figure legends

Fig. 1 Time course of viable cell density (A) and viability (B) of 1G3 recombinant insect cells treated with LiCl in static culture. (C) Time course of antigen-binding activity of scFv-Fc in the culture supernatant measured by enzyme-linked immunosorbent assay (ELISA). $A_{450-600}$, difference of absorbance at 450 nm and 600 nm.

Fig. 2 Time course of viable cell density (A) and viability (B) of 1G3 cells treated with LiCl on day 4 after inoculation in static culture. (C) Time course of antigen-binding activity of scFv-Fc in the culture supernatant measured by ELISA.

Fig. 3 Staining of autophagosomes (A) and nuclei (B) in 1G3 cells treated with LiCl and autophagy inhibitor bafilomycin A1. Autophagosomes and nuclei were stained with the Cyto-ID Autophagy detection kit.

Fig. 4 Time course of viable cell density (A) and viability (B) of 1G3 cells treated with LiCl in shake-flask culture. (C) Time course of scFv-Fc concentration in the culture supernatant measured by ELISA.

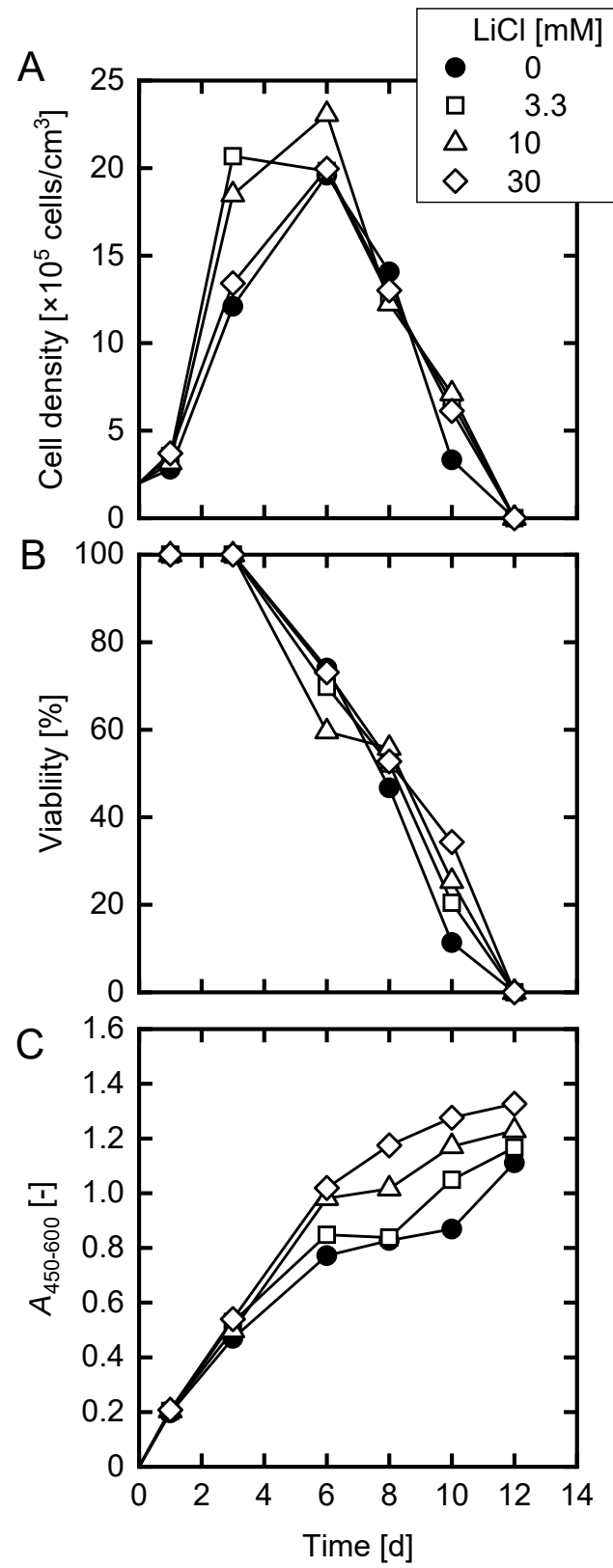


Fig. 1

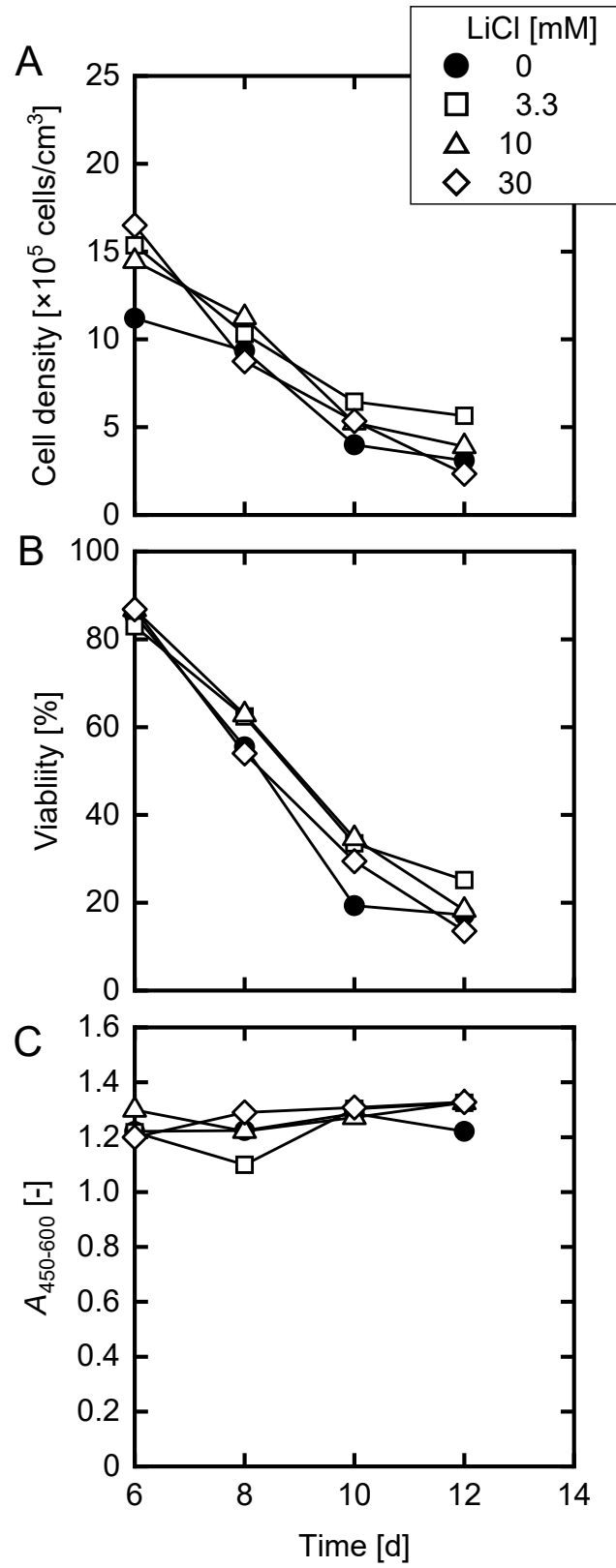


Fig. 2

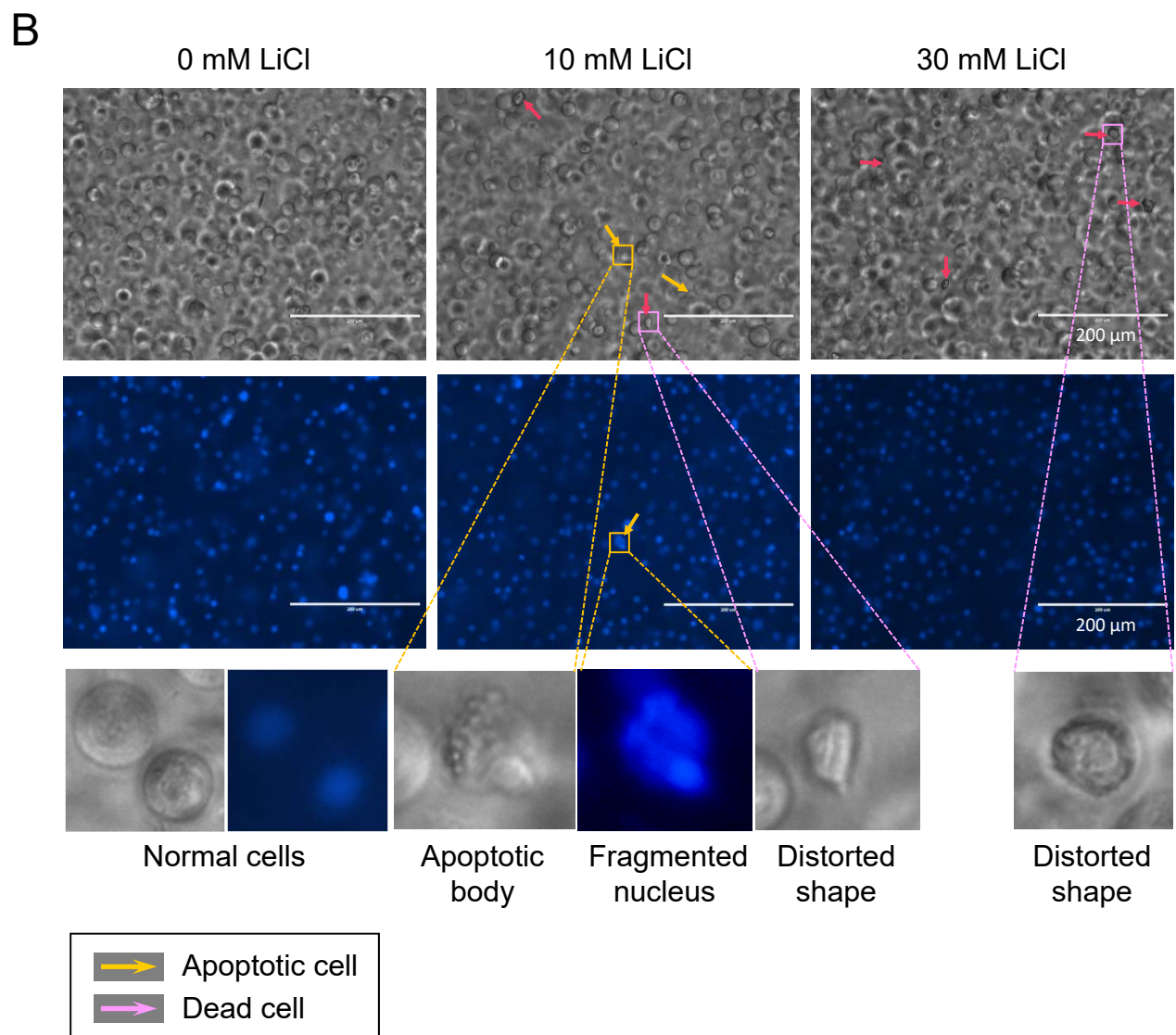
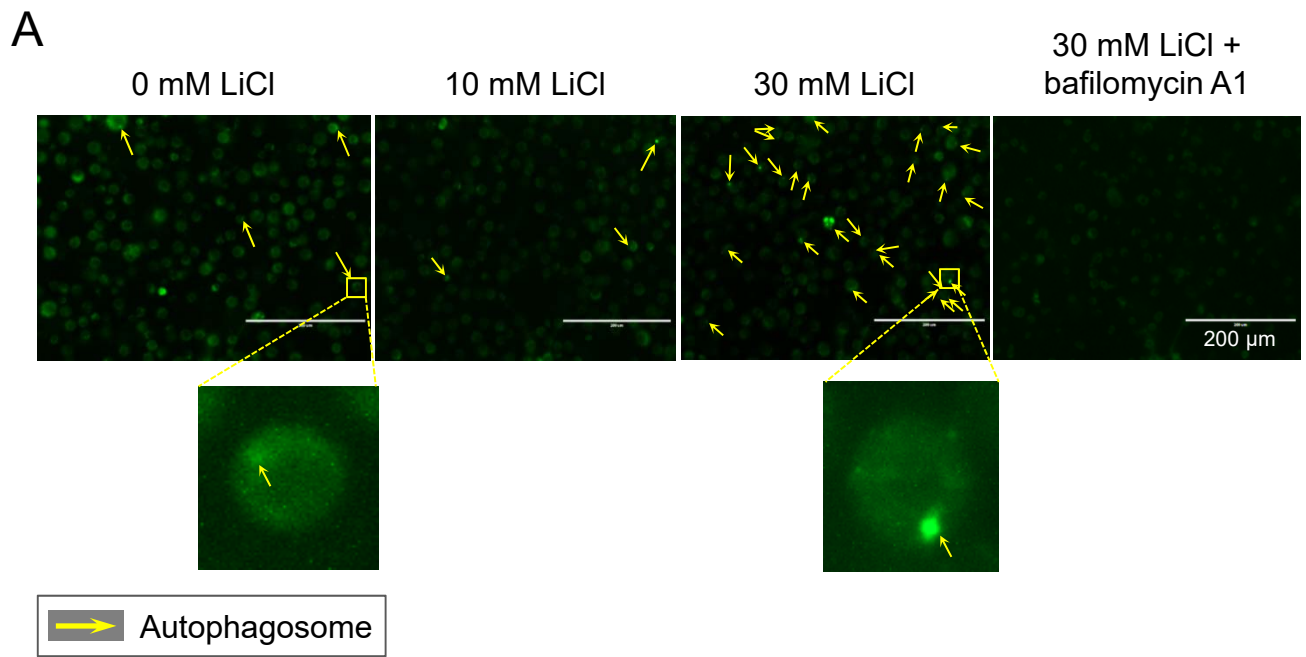


Fig. 3

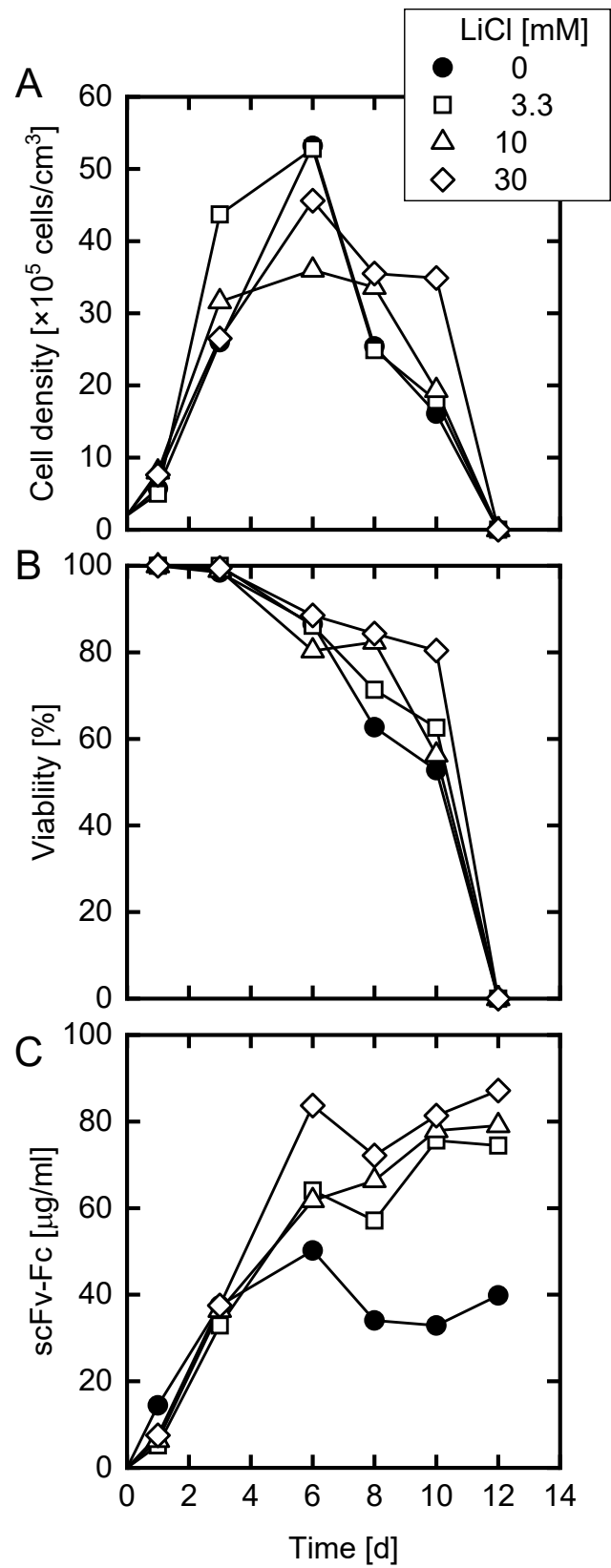


Fig. 4

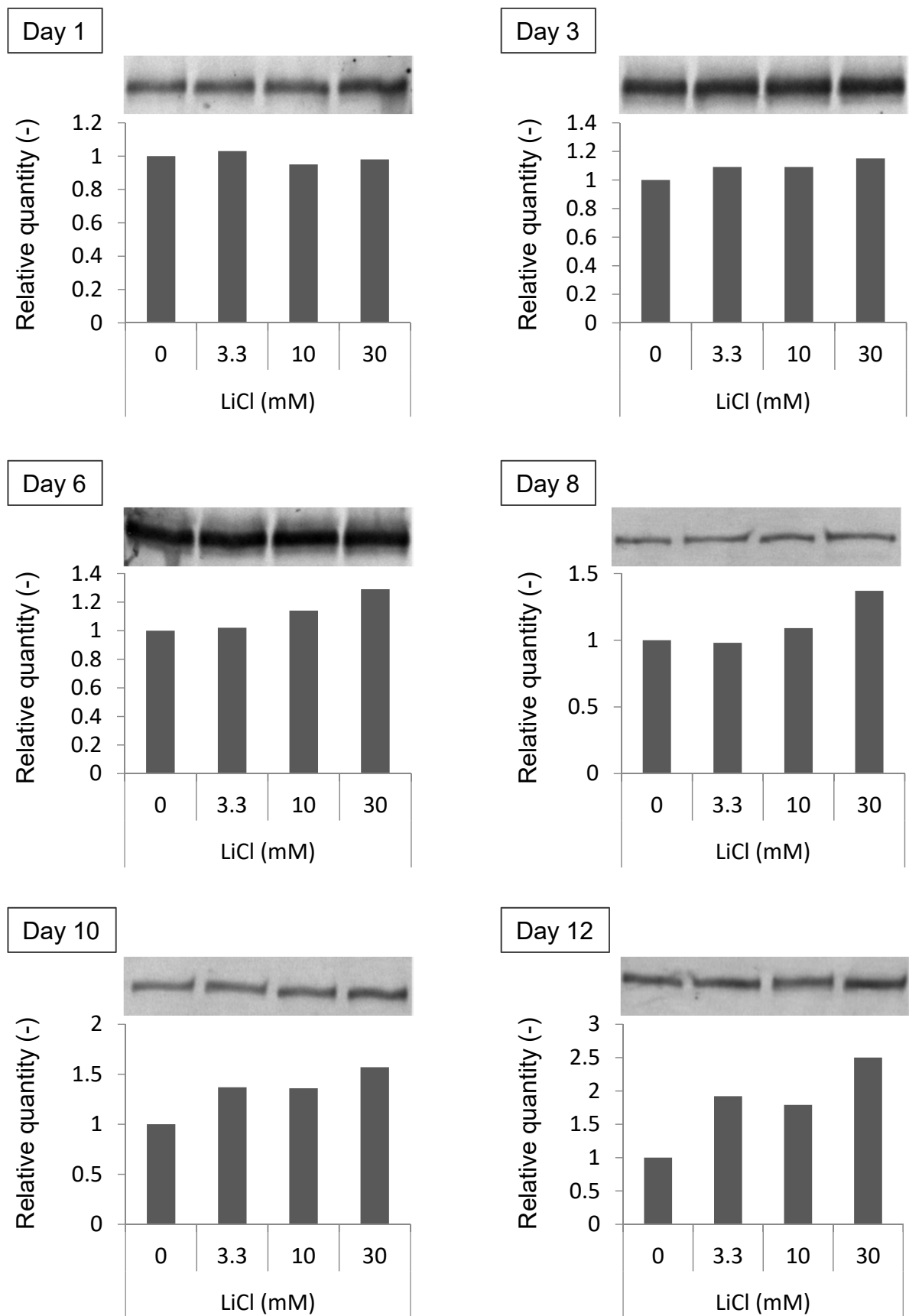


Fig. S1 Quantity of scFv-Fc secreted from 1G3 recombinant insect cells treated with LiCl in static culture. Specific bands were detected by western blotting, and the relative intensities of the bands were calculated. The quantity of scFv-Fc from cells without LiCl on each day is indicated as 1.

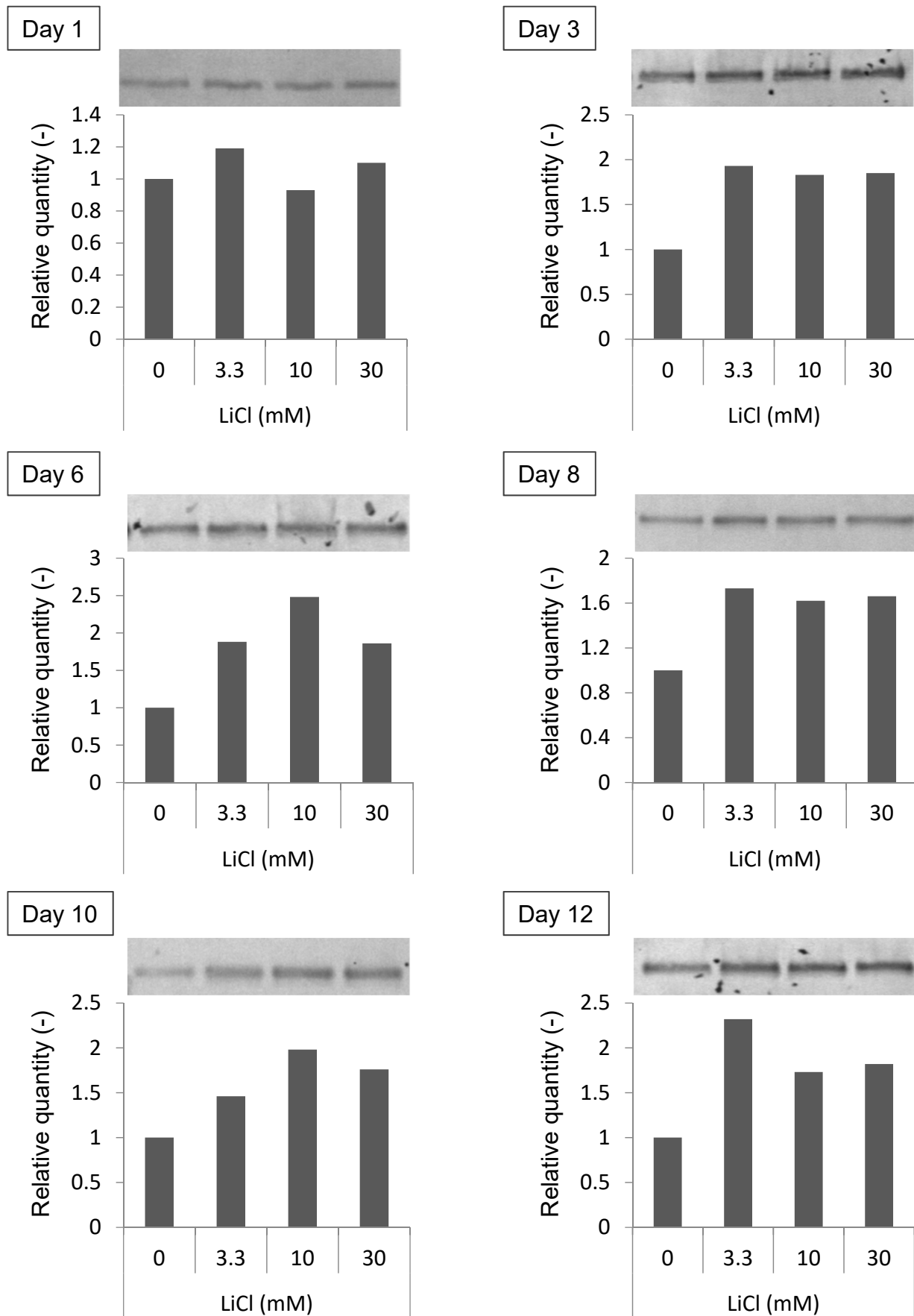


Fig. S2 Quantity of scFv-Fc secreted from 1G3 cells treated with LiCl in shake-flask culture. Specific bands were detected by western blotting, and the relative intensities of the bands were calculated. The quantity of scFv-Fc from cells without LiCl on each day is indicated as 1.