Autophagy-inducing Peptide Increases CHO Cell Monoclonal Antibody Production in Batch and Fed-batch Cultures

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September 14, 2020

Abstract

The development of generic biopharmaceuticals is increasing the pressures for enhanced bioprocess productivity and yields. Autophagy ("self-eating") is a cellular process that allows cells to mitigate stresses such as nutrient deprivation. Reputed autophagy inhibitors have also been shown to increase autophagic flux under certain conditions, and enhance recombinant protein productivity in Chinese Hamster Ovary (CHO) cultures. Since peptides are commonly added to bioprocess culture media in hydrolysates, we evaluated the impact on productivity of an autophagy-inducing peptide (AIP), derived from the cellular autophagy protein Beclin 1. This was analyzed in CHO cell batch and fed-batch serum-free cultures producing a human IgG1. Interestingly, the addition of 1 to 4 µM AIP enhanced productivity in a concentration-dependent manner. Cell-specific productivity increased up to 1.8-fold in batch cultures, while in fed-batch cultures a maximum 2-fold increase in volumetric productivity was observed. An initial drop in cell viability also occurred before cultures recovered normal growth. Overall, these findings strongly support the value of investigating the effects of autophagy pathway modulation, and in particular, the use of this AIP medium additive to increase CHO cell biotherapeutic protein production and yields.

Introduction

Mammalian cell cultures are widely used for the production of protein biopharmaceuticals because of their capacity for complex and physiologically relevant post-translational modifications, often required for therapeutic efficacy (Varki et al., 2009). Chinese Hamster Ovary (CHO) cells have become the most used cell line for industrial production of recombinant glycoproteins due to their in-depth characterization and capacity to produce human-like glycosylation (Hamaker and Lee, 2018; Hong et al., 2018).

In the past decades, there has been an increasing demand for large-scale production of biopharmaceuticals, especially antibodies, from mammalian cell culture processes (Lalonde and Durocher, 2017). More than 90% of all antibodies on the market are produced in mammalian cells (Ecker et al., 2014). Over the years, cell culture engineering has made significant advances with improved growth media, culture condition control and cell lines (Butler and Meneses-Acosta, 2012; Kelly et al., 2018). In media development, formulations based on cellular metabolic needs (Ghaffari et al., 2020), as well as the addition of small molecules such as butyrate (Mimura et al., 2001) or butyrated ManNAc (Yin et al., 2018), have increased recombinant protein production. Improvements in cell line engineering include the development of superior mammalian expression systems (Bebbington et al., 1992; Lucas et al., 1996; Hamaker and Lee, 2018; Amann et al., 2019; Bulté et al., 2020) and the inclusion of anti-apoptotic genes to improve cell survival (Kim and Lee, 2002; Mastrangelo et al., 2000; Tey et al., 2000; Figueroa et al., 2007) with the goal of enhancing specific and volumetric productivity.

While apoptosis caused by nutrient limitations is the most well-known cell death mechanism impacting bioprocesses (Kim et al., 2012), autophagy ("self-eating") is being studied in this context as well, often

functioning as a pivotal tipping point between cell survival and death (Doherty and Baehrecke, 2018). Indeed, through vacuolar engulfment, degradation, and recycling of large protein complexes to entire organelles, autophagy is a critical mitigating response to metabolic stressors including nutrient depravation, thereby maintaining the cell's energetic needs and homeostasis (Hwang and Lee, 2008; Zustiak et al., 2008; Mizushima and Klionsky, 2007; Mizushima et al., 2008; Doherty and Baehrecke, 2018). We previously reported that the addition of 3-Methyl Adenine (3-MA), a reputed autophagy inhibitor, increased recombinant protein productivity in CHO cell fed-batch cultures (Jardon et al., 2012). This phenomenon was further studied by Baek et al. (2016), testing 3-MA and eight other chemical inhibitors of autophagy, and their respective effects on cell culture productivity. An increased specific productivity was confirmed for 3-MA, and also observed in response to two other reputed autophagy inhibitors including dorsomorphin (AMP-activated protein kinase inhibitor), and SP600125 (c-Jun NH2-terminal kinase inhibitor). However, Baek et al. (2016) recognized that these agents did not inhibit autophagy but rather appeared to induce it, thereby linking 3-MA-enhanced autophagic flux rather than inhibition with increased bioprocess productivity. This induction of autophagy in response to 3-MA, particularly under nutrient-rich culture conditions, is consistent with previous studies by Wu et al. (2010).

In this study, a novel autophagy-inducing peptide was tested to further investigate the influence of this cellular process on secreted protein production. While the addition of chemicals to induce autophagy in cell culture is possible, their use comes with the risks of confounding effects (e.g. 3-MA (Klionsky et al., 2016, Wu et al., 2010)). The use of an alternative, more specific agent that could be more readily incorporated into an industrial bioprocess and at lower concentrations is preferable. Indeed, current media formulations often contain peptides as part of hydrolysates added to the medium to increase growth and productivity (Spearman et al., 2014). This made the use of a unique and specific cell-permeable, autophagy-inducing peptide (AIP), derived from the autophagy protein Beclin 1 (Shoji-Kawata et al., 2013), suitable for this purpose. Therefore, we evaluated and developed the capacity of this AIP to enhance therapeutic protein productivity in CHO cells maintained in batch culture as well as in the more industrially relevant fed-batch culture. In addition to therapeutic protein productivity, we determined the impact of the AIP on cell culture growth and viability.

Materials and Methods

Cell line and culture maintenance

The Chinese Hamster Ovary (CHO) cell line expressing human monoclonal anti-Interleukin-1β IgG1 (Kennard et al., 2009) ChK2 437.89.34 was provided by Pfizer (St. Louis, MO). The cell line was maintained in shake tubes (Corning or Nalgene) in a humidified incubator (Kuhner shaker, Basel, Switzerland) at 37@C, 5% CO_2 and 225 rpm. The cells were passaged every 3 days and grown in chemically defined CD CHO medium (Gibco-Invitrogen, Grand Island, NY), supplemented with 100 μg/mL hygromycin B (Gibco), 4.5 μg/mL bleocin (Calbiochem, La Jolla, CA) and 4 mM glutamine (Gibco).

Autophagy-Inducing Peptide (AIP)

The autophagy-inducing peptide (AIP) and negative control peptide were provided by Dr. Beth Levine (Howard Hughes Medical Institute and University of Texas Southwestern Medical Center, Dallas, TX), and based on her group's previous studies (Shoji-Kawata et al., 2013). The cell-permeable AIP was comprised of a portion of the Beclin 1 autophagy protein spanned by amino acids 267-284, joined via a diglycine linker to the HIV-1 Tat protein transduction domain (PTD) (Shoji-Kawata et al., 2013, van den Berg and Dowdy, 2011). The control peptide consisted of an identical Tat PTD domain linked to a scrambled Beclin 1 amino acid sequence that does not induce autophagy (Shoji-Kawata et al., 2013). Both peptides were reported to be well-tolerated in mice when administered *in vivo* (Shoji-Kawata et al., 2013).

Batch Induction Experiments

The batch induction experiments were run in 24-well deep well plates (Axygen Scientific, Union City, CA) using Duetz covers (Kuhner shaker). The cultures were seeded at 2 x 10^5 cells/mL in 3.5 mL CD CHO

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medium supplemented with 100 μg/mL hygromycin B, 4.5 μg/mL bleocin and 4 mM glutamine. Cultures were incubated at 37°C, 5% CO_2 and 225 rpm in a humidified incubator (Kuhner Shaker). The experiment was initiated by adding autophagy-inducing peptide (AIP) stock solution (1.65 mM) to the cultures to obtain the medium concentrations of 0, 1, 2, 3 and 4 μM. Every two days, a 300 μL sample was taken for monitoring of cell density, cell viability, cell diameter, and cell aggregation (Cedex analysis) using a Cedex automated cell counting instrument (Innovatis, Bielefeld, Germany), according to the manufacturer's instructions. Once cultures reached a cell density above 2×10^6 cells/mL, 100 μL of the culture sample were mixed with 200 μL of 1X Dulbecco's phosphate-buffered saline (DPBS) (Gibco) and used for cell count and viability measurements at a 1:3 dilution. The remainder of the culture sample was centrifuged to remove the cells and approximately 150 μL of the supernatant were retained to determine the IgG production by ELISA. Each AIP medium concentration was evaluated in duplicate cultures and the entire batch induction experiment was repeated two additional times (for a total of 3 times). In addition, a batch induction experiment was performed using the negative control peptide.

Fed-Batch Induction Experiments

The fed-batch induction experiments were performed in 24-well deep well plates (Axygen Scientific) using Duetz covers. The cultures were seeded at 2×10^5 cells/mL in 3.5 mL CD CHO medium supplemented with 100 μg/mL hygromycin B, 4.5 μg/mL bleocin, and 4 mM glutamine, and incubated at 37°C, 5% CO_2 and 225 rpm in a humidified incubator (Kuhner shaker). The experiment was initiated by adding the AIP stock solution (1.65 mM) to obtain medium concentrations of 0, 1, 2, 3 and 4 μM. Starting on day 1, the fed-batch regime was initiated with a 280 μL sample taken daily from the culture. This volume was then replaced with 140 μL CD CHO Efficient Feed A (Gibco), 70 μL amino acid mixture A [10 mM L-cystine disodium salt hydrate (MP Biomedicals, Illkirch, France), 15 mM L-tyrosine disodium salt (Sigma, St. Louis, MO) and 10 mM aspartic acid (Gibco) dissolved in 0.1 N HCl] and 70 μL amino acid mixture B [10 mM L-glutamic acid (Sigma) and 75 mM L-asparagine (Sigma) in 0.1 N NaOH]. Every two days, 150 μL of the removed culture volume was mixed with 150 μL of DPBS and used for Cedex analysis at a 1:2 dilution. As above, when cultures reached a concentration of greater than 2×10^6 cells/mL, a 100 μL aliquot was taken to determine cell count and viability at a 1:3 dilution with DPBS. Similar to the batch cultures, the remainder of the culture sample was centrifuged and the supernatants were retained for IgG quantitation by ELISA. Each AIP medium concentration was evaluated in duplicate cultures during a run and the entire fed-batch induction experiment was repeated (for a total of 3 times).

Cell count and viability determination

Viable cell concentration was measured by trypan blue exclusion using a Cedex automated cell counting instrument, according to the manufacturer's instructions (Innovatis). Measurements were performed every two days during the culture period using 0.4% (w/v) trypan blue (Gibco).

Recombinant protein quantification

Levels of IgG1 in culture supernatants were evaluated by ELISA. Standard ELISA protocols were followed using 96-well Maxisorb plates (Thermo Fisher Scientific) and signals measured using a plate reader (Molecular Devices, Sunnyvale, CA) at 405 nm (reference at 492 nm). The primary (coating) antibody used was a goat anti-human IgG (Fcγ fragment-specific) (Jackson ImmunoResearch #109-005-098). The secondary (detection) antibody used was a goat anti-human IgG (H+L specific)-alkaline phosphatase antibody (Jackson ImmunoResearch #109-055-088). Quantification was achieved by interpolation from a standard curve obtained from 1.9 - 125 ng/mL dilutions of purified IgG.

The volumetric productivity was determined by the overall production of IgG for each culture volume, while the specific productivity for each culture was calculated based on the product concentration over the integral viable cell density (IVCD) (Renard et al., 1988).

Statistical analysis

Differences between measurements was evaluated by one-way ANOVA followed by multiple comparison tests (Tukey-Kramer HSD Post-Hoc test). P values of < 0.05 were considered statistically significant.

Results and Discussion Autophagy enhances recombinant antibody production in batch cultures

Our initial investigations evaluated whether addition of the autophagy-inducing peptide (AIP), derived from the autophagy protein Beclin 1 (Shoji-Kawata et al., 2013), could enhance monoclonal antibody production in batch cultures. CHO cells expressing a human anti-IL1β IgG1 (CHO-aIL1β) were cultured in medium supplemented with AIP at 0, 1, 2, 3 and 4 μM concentrations, and IgG production was evaluated by ELISA, as described in Materials and Methods. Figure 1A compares the specific and volumetric productivities in the control- and AIP- treated cultures from a representative batch induction experiment. The specific productivity for the AIP-treated cultures increased from 11.0 ± 0.9 to 19.2 ± 0.7 μg/10^6 cells/day at 1 and 4 μM concentrations, respectively. The specific productivities observed for the 2, 3, and 4 μM AIP-containing cultures were significantly increased (ANOVA, $P < 0.05$) relative to the control culture (10.6 ± 0.6 μg/10^6cells/day). Similarly, the volumetric productivity increased from 404.9 ± 28.3 at 1 μM AIP to 565.5 ± 39.6 μg/mL at 4 μM AIP. However, the increase in volumetric productivity was statistically significant only for the 4 μM AIP concentration, as compared to the control culture (ANOVA, $P < 0.05$). Thus, substantial increases were observed, up to 82% and 46% in specific and volumetric productivities, respectively.

Peptide hydrolysates have been added to cell culture manufacturing media to increase cell growth and productivity (Franek et al., 2003; Franek and Fussenegger, 2008). To determine whether the impact of the AIP was indeed due to its capacity to induce autophagy, a negative control peptide containing the same amino acids, but in a scrambled arrangement, was also tested. Figure 1B shows that 3 or 4 μM concentrations of the negative control peptide had little if any influence on the protein productivity compared to the control culture containing 0 μM peptide. Furthermore, it was confirmed that the addition of the AIP to the culture medium induced cellular autophagy in CHO-aIL-1β cells, while the control peptide did not, as evaluated by cellular p62 protein degradation in Western blot analyses (Shoji-Kawata et al., 2013 and data not shown). Therefore, the increase in productivity observed was linked to the induction of autophagic flux in the cell culture, and this is consistent with our previous findings (Jardon et al., 2012) as interpreted and confirmed by Baek et al. (2016). Shoji-Kawata et al. (2013) have shown that the AIP used in these experiments specifically triggers the autophagy cascade by promoting the release of Beclin 1 from one of its inhibitors, the Golgi-associated plant pathogenesis-related protein 1 (GAPR-1). However, the molecular mechanism by which autophagic flux, in response to the AIP, leads to enhanced protein productivity in culture supernatants is unclear at present. It is interesting to note that the increased autophagy and CHO cell productivity in response to 3-MA was associated with the induction of the unfolded protein response (UPR) at the transcriptional level (Baek et al. 2018). These authors further suggested that UPR induction may enhance endoplasmic reticulum (ER) capacity and thereby the cellular productivity, a possibility that requires further investigation.

The effect of the autophagy-inducing peptide on cell growth and viability in batch culture

Given the encouraging productivity results, it was important to consider the effects of the AIP on the growth and viability of the CHO cells in culture. The results obtained with the AIP were compared to two control cultures containing either no peptide or the scrambled negative control peptide that did not induce autophagy (Figure 1B). Compared to the two control cultures, with the addition of increasing AIP concentrations, there was an increasing lag in growth over the first 4-8 days of culture (Figure 2A). Subsequently, the AIP-containing cultures had similar rapid growth phases, resulting in a ~5 day extended batch culture duration. These cultures reached maximum cell densities from 8.3 ± 0.1 to 9.9 ± 0.6 x 10^6 cells/mL, with Integral Viable Cell Density (IVCD) values from 26.2 ± 0.3 to 40.9 ± 0.3 x 10^6 cells-day/mL. After reaching the maximum, the viable cell densities consistently declined rapidly over the ensuing 2 days. Notably, the addition of 2-4 μM AIP to the cultures resulted in significantly lower (ANOVA, $P < 0.05$) maximum cell densities and IVCDs compared to the control and 1 μM AIP-containing cultures. These differences, along with the more pronounced lag phases at higher AIP concentrations, are likely the result of the complex control exerted over

cell survival and death by the autophagy pathway (Doherty and Baehrecke, 2018).

The batch culture viability profiles are shown in Figure 2B. The control cultures maintained a >90% viability until day 6, before a rapid viability decline. For all cultures treated with AIP, the lag in growth was accompanied by an initial drop in viability observed on day 2. Similarly, the extent of the drop was AIP concentration dependent, with 4 μM of AIP exerting the largest, >90% decrease in cell viability. This decrease in viability was not observed in the control peptide culture, nor would it be expected from peptide hydrolysate medium supplementation. Also, the decrease in viability was not attributable to the protein transduction domain of the tat motif present in both peptides to facilitate their uptake into the cell (Herce and Garcia, 2007). The day 2 decreased viability likely resulted from the induction of autophagic cell death or "autosis", which can be a consequence of autophagic flux in response to the AIP, as previously reported (Liu et al., 2013). Interestingly however, the viability in all AIP-treated cultures recovered rapidly after day 2, reaching over 90% by day 6. After day 8, another rapid decrease in viability was observed as these batch cultures entered the decline phase. It appears that the autophagic flux in response to the AIP, the ensuing cell death, and rapid subsequent expansion of surviving cells, creates conditions and/or selects for cells that have superior protein production capabilities. This merits further investigation, especially in the context of the fed-batch processes most commonly used for monoclonal antibody manufacturing.

Induction of the autophagy pathway enhances antibody productivity in fed-batch cultures

Since the most common culture systems found in industry are fed-batch cultures, the ability of the AIP to enhance protein production was also evaluated on this culture platform. CHO-aIL-1β cells were cultured in the presence or absence of AIP using fed-batch and the production of IgG was evaluated by ELISA. Figure 3 shows the specific and volumetric productivity in AIP-treated and control (0 μM peptide) cultures. The specific productivity increased from 9.6 ± 1.5 μg/10^6cells/day in control cultures up to 13.7 ± 0.6 μg/10^6cells/day in AIP-treated cultures. The increase in specific productivity above that of controls was statistically significant in 2 and 4 μM AIP-containing cultures (ANOVA, $P < 0.05$). The volumetric productivity also increased significantly (ANOVA, $P < 0.05$) to 1260.1 ± 87.1 and 1229.7 ± 90.0 μg/mL in cultures with 3 and 4 μM AIP concentrations, so by over 2-fold compared to the control cultures. These findings are consistent with those obtained in batch cultures, but with the volumetric productivity increase over twice as much as the 46% increase observed in batch culture.

Growth and viability of fed-batch cultures in response to AIP

As in the batch cultures, we investigated the effects of AIP on the growth and viability profiles of CHO-aIL-1β cells in the fed-batch cultures. AIP-treated cells were compared to the 0 μM AIP control (Figure 4). Both the control cultures and those stimulated with 1 μM AIP grew relatively rapidly with similar kinetics before slowing and reaching a maximum by day 10, and then rapidly dropping off thereafter. As in the Figure 2A batch cultures, correlated with higher additions of AIP, an increasing lag in growth was observed (Figure 4A). After this initial lag phase, the 2, 3 and 4 μM AIP-containing cultures grew rapidly for an additional 6 days before reaching a maximum, with 3 and 4 μM AIP cultures exhibiting a stationary phase before declining. Compared to the control (20.2 +/- 1.7 x 10^6 cells/mL), significantly lower maximum cell densities (ANOVA, $P < 0.05$) were observed at AIP concentrations of 3 μM (13.4 +/- 0.9 x 10^6 cells/mL) and 4 μM (10.3 +/- 0.7 x 10^6 cells/mL). Concomitantly, for the 3 and 4 μM AIP cultures, the IVCD values were significantly increased (ANOVA, $P < 0.05$) compared to the control, 1 μM, and 2 μM cultures, indicating lower maximum growth but increased culture longevity.

The viability profiles for all cultures are shown in Figure 4B. Control and 1 μM AIP-containing cultures maintained viabilities >90%, until they decreased after day 10. Cultures containing 2 - 4 μM AIP exhibited an initial and AIP dose-related drop in viability by day 2. In particular, both the 3 and 4 μM AIP cultures had viabilities of less than 50% by culture day 2. Similar to the batch cultures, a rapid recovery in viability for all AIP cultures was observed on day 4. The cell viability remained above 90% after day 6 until cultures reached their maximum cell concentration, before declining again. After day 10, a rapid decline in viability was observed for control, 1 and 2 μM cultures, while 3 and 4 μM cultures had a more prolonged decline

phase. Overall, as for the batch cultures, the initial decline in cell viability could be due to autophagic death, and then the autophagy induction may promote the subsequent rapid recovery and altered growth characteristics (prolonged stationary phase), either selecting cells or providing the conditions that enhance recombinant protein productivity.

Conclusion

Overall, this study provides strong support for modulating cellular autophagy to increase monoclonal antibody production in the CHO cell culture system, both in batch and fed-batch cultures. Despite the negative impacts on cell growth and viability (that could be reduced), the positive impacts on both cell specific and volumetric antibody production were substantial. This unique peptide and its capacity to specifically activate the autophagy cascade, merits further consideration as a media additive for bioprocess refinement, and is thus of particular relevance to industrial biotechnology.

Acknowledgements

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Figure Legends

Figure 1. A) The effect of AIP on the specific and volumetric productivity of aIL-1β-producing CHO cells in a batch culture compared to control cultures containing no added peptide. Error bars indicate the standard deviation for two technical replicate cultures in one experiment, assuming that the data is normally distributed. These data are from one representative run out of three exhibiting the same trend. B) The effect of a negative control peptide (NCP) on the specific and volumetric productivity in CHO-aIL-1β cells batch culture with 3 µM and 4 µM NCP added to the basal cell culture medium on day 0.

Figure 2. The effect of autophagy on the A) growth profiles and B) viability for aIL-1β-producing CHO cells in batch cultures over time. Cultures with no added peptide (Control) or with the negative control peptide (NCP 3 µM) were experimental controls. The AIP concentrations tested were 1 µM, 2 µM, 3 µM, and 4 µM. Error bars indicate the standard deviation for two technical replicate cultures within one run, assuming that the data is normally distributed. These data are from one representative run out of three exhibiting the same trend.

Figure 3. The effect of AIP (1-4 µM) on the specific and volumetric productivity of aIL-1β-producing CHO cells in fed-batch cultures compared to the control cultures containing no additional peptide. Error bars indicate the standard deviation for two technical replicate cultures within one run, assuming that the data is normally distributed. These data are from one representative run out of three exhibiting the same trend.

Figure 4. The effect of AIP on the A) growth profiles and B) viability of aIL-1β-producing CHO cells in fed-batch cultures. Cultures without AIP were used as experimental controls. Error bars indicate the standard deviation for two technical replicate cultures within one run, assuming that the data is normally distributed. These data are from one representative out of three exhibiting the same trend.

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