Survival Factor-Like Activity of Small Peptides in Hybridoma and CHO Cells Cultures

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Synthetic peptides containing three to six amino acid residues were previously shown to improve key parameters of monoclonal antibody-producing mouse hybridoma cultures. The aim of the current work was to investigate whether small peptides also exert analogous beneficial impact on a CHO-K1-derived cell line (XMK-111-10) engineered for production of the human model glycoprotein SEAP (secreted alkaline phosphatase). Similar to hybridoma cultures, growth and SEAP production profiles of CHO XMK-111-10 were modulated by peptides. Both viable cell density and SEAP production were increased by tetraalanine or by a fraction of wheat gluten hydrolysate. Whereas tetraglycine increased the peak viable cell density, the growth-suppressing tripeptide Gly-Lys-Gly significantly boosted SEAP production. All peptide-supplemented cultures showed slight improvement of culture viability during the decline phase of the batch cultures, suggesting a survival factor-like activity of the peptides.

Introduction

Animal cells cultured in protein-free media are sensitive to suboptimal nutrient levels, which induce programmed cell death known as apoptosis (*1*−*3*). The onset of apoptosis results in premature termination of the cultures and in low product yields. Previous work has shown that the decay of cultures may be suppressed or delayed by specific medium additives, such as peptones (*4*, *5*) or pure peptides (*6*, *7*).

The favorable effects of peptones or peptides cannot be interpreted as a result of optimization of metabolic substrates levels, because

(1) The amino acid composition of some of the active supplements is rather unbalanced, and essential amino acids are not sufficiently represented (*8*).

(2) Pure synthetic peptides, containing three to six amino acid residues, may improve key culture parameters, even if such peptides are composed of nonessential amino acids (*6*).

The effects of synthetic pure peptides on hybridoma cultures are summarized by the following points (*6*, *7*, *9*):

(1) The effects of peptides increase with peptide chain length up to the length of pentapeptides. The effects of peptide-constituting amino acids in free form are different from those of the peptides (i.e., an opposite effect or no significant effect).

(2) The population of active peptides is rather heterogeneous. The population of these peptides contains neither any common specific amino acid nor any common sequence motif.

(3) Some peptides exclusively increase peak cell density (e.g., Gly-Gly-Gly-Gly, Gly-Phe-Gly), whereas others improve peak cell density and product yield (e.g., Ala-Ala-Ala-Ala, Ser-Ser-Ser, Thr-Thr-Thr) or suppress growth and enhance the product yield (e.g., Gly-Lys-Gly, Lys-Lys-Lys, Gly-His-Lys).

(4) The general effect of all peptides is increased cell viability.

(5) Peptides are effective at concentrations above 1 mM.

(6) The peptides are relatively stable in cell cultures. Utilization of liberated amino acids is marginal.

(7) In the presence of peptides the distribution of cell-cycle phases may be altered.

The aim of this work was to investigate the effects of selected peptide representatives on a cell line of non-lymphoid tissue origin. The experiments carried out using the CHO-K1-derived SEAP-producing cell line XMK-111-10 indicated strong analogy to the mode of peptide action previously shown for mouse hybridomas.

Materials and Methods

Materials. Cell culture media and supplements were purchased from Sigma-Aldrich (St. Louis, MO). Synthetic peptides were purchased from Bachem (Bubendorf, Switzerland) and from PolyPeptide Laboratories (Prague, Czech Republic). The chromatography fraction a21 of wheat gluten enzymic hydrolysate was prepared as described previously (*8*).

Cell Culture. Mouse hybridoma ME-750 was cultured in DMEM/F12/RPMI 1640 (3:1:1) medium supplemented with BME amino acids, 2.0 mM glutamine, 0.4 mM of each of the amino acids alanine, serine, asparagine, and proline (*10*), 15 mM HEPES, and 2.0 g L^{-1} sodium bicarbonate and the iron-rich, protein-free, growth-promoting mixture containing 0.4 mM ferric citrate (*11*).

SEAP-producing CHO XMK-111-10 (*13*) was cultured in Iscove's DMEM/F12/RPMI 1640 (2:1:1) medium supple-

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mented with 2.0 g L^{-1} sodium bicarbonate and the iron-rich, protein-free growth-promoting mixture containing 0.5 mM ferric citrate (*11*).

The cultures were kept in 25 cm^2 T-flasks at 37 °C in a humidified atmosphere containing 5% CO_2. The culture volume was 6.0 mL. In fed-batch cultures, a volume of 0.25 mL of a feeding solution (DMEM fortified with 10× BME amino acids, 10× BME vitamins, and 20 mM glutamine) was added daily starting from day 1. Viable and apoptotic cells were counted using a hemocytometer. Apoptotic cells were identified by apoptotic morphology, i.e., shrunken cells with ruffled membranes, and were distinctive from the round-shaped live cells. No swollen necrotic cells were observed in static cultures used for the assays. A total of 500 cells were counted in each flask. The assays were conducted in triplicate. The standard deviation involved in the quantitation of cell density and viability was ±10%.

Assays of Peptide Activity. The hybridoma cultures were inoculated at a density of $(250 \pm 50) \times 10^3$ cells mL^{-1} and incubated until the decline phase, i.e., for 7 days. The monoclonal antibody (MAb) concentration in hybridoma culture supernatants was determined by immunoturbidimetry (*12*). Briefly, aliquots of media were diluted by 5% poly(ethylene glycol) and incubated for 1 h with porcine anti-mouse immunoglobulin affinity purified antibody. The turbidity was measured at 340 nm, and the MAb concentrations were determined using a calibration curve. The standard deviation associated with the quantitation of MAb concentrations was ±5%.

The XMK-111-10 cultures were inoculated at a density of $(100 \pm 40) \times 10^3$ cells mL^{-1} and incubated for 8 days. SEAP activity in CHO XMK-111-10 culture supernatants was determined after incubation with *p*-nitrophenyl phosphate (*13*). *p*-Nitrophenol liberated by the enzyme was determined by absorbance at 405 nm. The standard deviation of triplicate SEAP production profiles did not exceed 5%.

Results and Discussion

The choice of tetraalanine for the basic comparison of peptide effects on hybridoma and on CHO XMK-111-10 cultures was based on the knowledge that the nonessential amino acid alanine was known to be produced by cultured animal cells even under conditions of limited starvation (*14*, *15*). Moreover, the balance of the sum of tetraalanine and alanine concentrations after a 4-day culture period has been found to be virtually zero (*6*). These facts excluded that alanine liberated from the tetrapeptide would act as an additional substrate and thus improve culture parameters.

In our exploratory studies tetraalanine was classified as a peptide enhancing both peak cell density and MAb production (*6*). The growth curves of 7-day hybridoma cultures, carried out in batch and fed-batch regimens, convincingly documented this classification (Figure 1, Table 1). The increased peak cell density in the presence of tetraalanine was obviously caused by a slight extension of the exponential phase. As evidenced by the viable cell density curves the growth rate at the exponential phase was not affected by the presence of the peptide. In the fed-batch culture a viable cell density exceeding 3×10^6 cells mL^{-1} was reached. The final MAb concentration in the fed-batch control culture (48 mg L^{-1}) represented 133% of the concentration in the batch control culture (36 mg L^{-1}). The MAb concentrations in the tetraalanine-supplemented cultures were higher, i.e., 44 mg L^{-1} in the batch culture and 60 mg L^{-1} in the fed-batch culture.

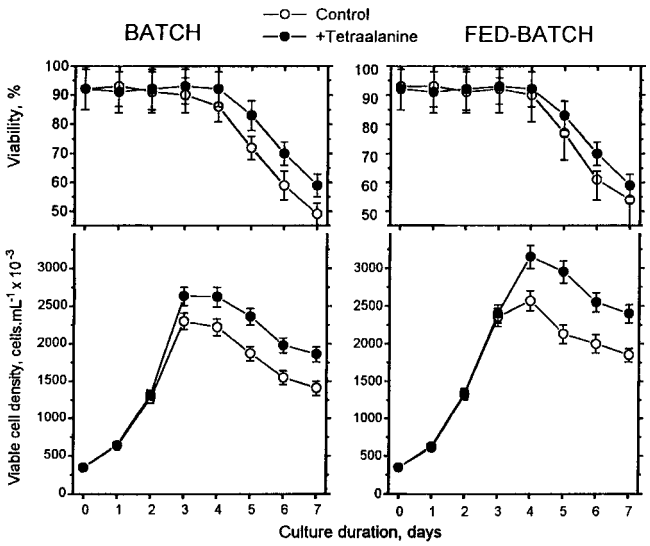

Figure 1. Batch culture and fed-batch culture of hybridoma ME-750 in the presence of 0.1% (w/v) tetraalanine. Error bars represent the standard deviation. The culture volume was 6.0 mL. A volume of 0.25 mL of a feeding mixture (see Methods) was added to the fed-batch culture daily starting from day 1.

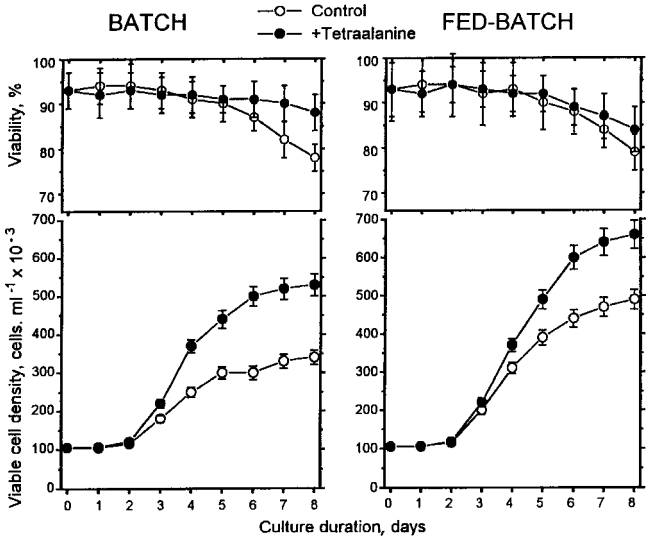

Figure 2. Batch culture and fed-batch culture of CHO XMK-111-10 cells in the presence of 0.1% (w/v) tetraalanine. Error bars represent the standard deviation. The culture volume was 6.0 mL. A volume of 0.25 mL of a feeding mixture (see Methods) was added to the fed-batch culture daily starting from day 1.

Table 1. Enhancement of Final Product Concentrations in Cultures Supplemented with 0.1 % Tetraalanine

	final product concentrations relative to control batch culture (%)	
cell line	batch mode	fed-batch mode
hybridoma ME-750	122	167
XMK-111-10	117	134

Thus, the effects of tetraalanine along with batch feeding with a medium concentrate appeared to be additive in nature. The culture viability at the stationary phase was slightly higher in tetraalanine-supplemented cultures.

The growth curves of CHO XMK-111-10 cultures demonstrated that the presence of tetraalanine influenced this CHO derivative in a similar way as has been observed with hybridoma cultures (Figure 2, Table 1). The peptide also increased the exponential growth rate. At the end of 8-day cultures the SEAP activity in the medium of the fed-batch culture reached 134% of levels

Table 2. Key Parameters of 8-Day CHO XMK-111-10 Batch Cultures Supplemented with Various Peptides

peptide	viable cells: $\times 10^{-3}$ cells mL^{-1}	viable cells: % control	viability (%)	SEAP activity (% control)
none (control)	410	100	78	100
tetraglycine				
0.2%	590	144	90	104
0.1%	680	166	89	92
0.05%	470	115	80	99
Gly-Lys-Gly				
0.2%	370	90	84	163
0.1%	400	98	87	160
0.05%	390	95	89	154
fraction a21				
0.2%	520	127	85	158
0.1%	530	129	84	162
0.05%	500	122	80	149

in the batch control culture. Also, tetraalanine slightly increased the culture viability at the stationary phase.

To characterize the effects of other categories of active peptides (*6*−*9*), we supplemented CHO XMK-111-10 cultures with (a) one of the peptides exclusively increasing the peak viable cell density, i.e., tetraglycine, (b) one of the peptides enhancing solely the final product yield, i.e., the tripeptide Gly-Lys-Gly, and (c) with the most potent fraction a21 isolated from wheat gluten enzymic digest by chromatography (*8*). The results of these experiments (Table 2) demonstrated that the impact of those peptides on CHO XMK-111-10 cultures was similar to that in hybridoma cultures (*6*, *7*, *9*). Viable cell density was higher in cultures supplemented with tetraglycine and with the fraction a21, whereas SEAP production was higher following supplementation with Gly-Lys-Gly or with fraction a21. Similar to the findings obtained with hybridoma cultures, the peptide-supplemented XMK-111-10 cultures showed higher viability.

Conclusions

CHO-derived recombinant cell lines are most often used for biopharmaceutical manufacturing of important protein therapeutics. This study confirms the positive impact of specific well-defined peptide additives on growth characteristics, cell viability, and overall production of CHO-K1-derived production cell line, analogous to findings obtained with hybridomas. This indicates that small peptides obviously hit some cellular targets present in many, if not all, mammalian cells. The present results provide an optimistic perspective for the use of small synthetic peptides as well-defined media additives to improve the performance of engineered mammalian cell lines.

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