学 位 論 文 の 要 旨

抗体産生における高生産性-高品質の株と低産生性-低品質の株との比較：培養工学的，生物物理学的，メタボローム的アプローチ

Comparison of monoclonal antibody-expressing cell lines with high productivity/high quality vs. with low productivity/low quality: approaches from fermentation engineering, biophysical chemistry, and metabolomic analysis

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抗体医薬品はその高い抗原特異性・長い血中半減期・低い想定外副作用の発生率から広く用いられるようになった．実際に，抗体医薬の市場は世界的に年々成長を続けている．治療用抗体は高い用量の投与が必要なこと，大腸菌とは異なり培養コストの高い動物細胞で発現する必要があることから，生産性の高い製造プロセスが患者の負担軽減のために必要である．一方，培養時のタンパク質の品質は精製工程を経ることもあり，これまでは，生産性ほど重要視されてこなかった．しかし，生産性の向上に伴い，培養時のタンパク質の品質が製品の品質に与える影響も無視できなくなってきた．培養液中の抗体品質の改善は，プロセスコストの削減だけでなく，副作用などの安全性からも重要である．モノクローナル抗体の生産性と品質は使用する細胞株の性質に大きく依存することから，抗体医薬品の開発工程において，大規模生産（商業生産）に適した細胞株を選択する工程は特に重要であると考えられている．本研究では，抗体産生で最も一般的に使用されるチャイニーズハムスター卵巣細胞（CHO 細胞）について，大規模生産に適した高い品質と高い生産性を有する細胞株の特性を明らかにすべく，種々の検討を行った．

まず第一に，高い生産性と低い凝集体含量を示す細胞を特徴づけるために，生産性の指標である抗体濃度，および凝集体含量の指標である高分子種の割合（HMWS(%)）とに密接に関係する因子を決定した．トラスツズマブ（商品名 ハークローン，ハーセプチン）を産生する 28 種の安定発現株を調製し，抗体濃度・培養液中の HMWS(%)などを分析した．抗体濃度および HMWS(%)の指標と様々な因子との関係を明らかにするために，ステップワイズ多重回帰分析を行った．その結果，高い抗体濃度は，高い比増殖速度（μ）・高い比生産速度（Q_p）・低い細胞内重鎖（HC）タンパク質含量と関係していることが明らかとなった．一方，低い HMWS(%)は，低い protein disulfide isomerase の mRNA レベル・高い培養液中の低分子量種の割合・高い Ｑｐ・高い細胞内軽鎖（LC）タンパク質含量・高いμと関係していることが明らかとなった．この結果は，ER 内での正しく，効率的な抗体分子のアッ

センブリーとフォールディングが，高い抗体濃度と低い凝集体含量にとって重要であることを示唆するものであった．

第二に，大規模生産に適した品質の抗体を産生する細胞の特性を明らかにするために，抗体サンプルおよび細胞の特性が，生産性および凝集体含量で対照的な細胞株間で異なるかどうかを調査した．28 株の中から細胞株 A（高い生産性，低い凝集体含量）および細胞株 B（低い生産性，高い凝集体含量）を選択し，それぞれの細胞株を 3 回培養した．細胞，それらの細胞によって生産された抗体分子（モノマー），そして凝集体の主要な性質の比較を行った．その結果，種々の差（増殖能，非共有結合性の凝集体含量，細胞内および培養液中 LC 含量，細胞内 HC および HC ダイマーの蓄積，非フコシル化の割合等）が存在することが明らかとなった. 細胞株 B での高い凝集体含量と低い抗体濃度は LC の低い生産性，およびそれに伴う多くの HC ダイマーとモノマーの蓄積に起因すると考えられる．また，凝集体含量だけでなく，凝集体形成の主要なメカニズムも 2 つの細胞間で異なっていた．細胞株 A 由来の凝集体は，主に共有結合性の相互作用により形成されるが，細胞株 B 由来の凝集体は，主に疎水性の相互作用により形成されることが明らかとなった．

第三に，メタボローム解析を用いて細胞株 A および細胞株 B の比較を行った．先に，細胞株 A と B の間の生産性（抗体濃度）の違いは，主に細胞数の違い（細胞株 A > 細胞株 B）であることが観察されている． 2 つの細胞株から分泌された抗体の品質において，抗体の抗体依存性細胞傷害活性と関連する非フコシル化されたオリゴ糖の割合に細胞のタイプで差が存在する（細胞株 A < 細胞株 B）ことが認められている．さらに，先の検討では，全凝集体含量および共有結合性凝集体の割合が細胞株 A と細胞株 B とで異なることを認めている．これらの違いが細胞代謝状態と関係しているか検討を行った．その結果，乳酸代謝シフトは高い生産性の細胞株を選択するのに有用であるが，細胞株 A での高い抗体濃度は乳酸代謝シフトが原因ではないことが示唆された．また，高い生産性と低い生産性の細胞株間で認められた増殖能の違いは，増殖期における細胞内の TCA サイクル中間体レベルの違いに起因することが示唆された．フコシル化オリゴ糖の割合の違いは，GDP-フコースの細胞内プールのレベル違いによるものではなく，フコシルトランスフェラーゼの発現レベルとその局在化の違いが原因ではないかと推定された．細胞株間での共有結合性の凝集体の割合の違いは，おそらく酸化ストレス状態の違いに起因しており，ミトコンドリアの酸化還元状態と関連している可能性が高いと推定された．

本研究の培養工学的・生物物理学的・メタボローム的アプローチにより，高生産性-高品質の抗体発現株と低産生性-低品質の抗体発現株との間に様々な違いが存在することが明らかとなった．本知見は，高生産性-高品質の抗体発現株の選抜・作成だけでなく，抗体の製造工程の改善にも役立つと期待される．

Comparison of monoclonal antibody-expressing cell lines with high productivity/high quality vs. with low productivity/low quality: approaches from fermentation engineering, biophysical chemistry, and metabolomic analysis

Therapeutic monoclonal antibodies (mAbs) have been widely used because of their high antigenic specificity, long serum half-life, and low incidence of undesirable side effects. In fact, the global market of therapeutic mAbs is growing annually. Because large doses of therapeutic mAbs are usually required and mAbs are commonly expressed in animal cells, which require high manufacturing cost compared with that of *E. coli*, mAb production processes with high productivity (titer) have been considered of prime importance in order to reduce expense to patients. On the other hand, since many of product-related impurities such as aggregates in culture media can be removed during purification processes, the quality of mAbs in culture media has not been regarded as an important issue compared with the productivity of host cell until recently. The influence of mAb quality in culture media on the drug product quality, however, cannot be disregarded in accordance with the recent increase in mAb productivity of host cell. The quality improvement of mAb in culture medium is important not only for process cost reduction but for the safety enhancement of the drug product. Because the productivity and quality of mAbs depend on cell lines employed, the selection of cell lines suitable for large-scale production (commercial manufacturing) is a very important step in process development for mAb production. In this study, I revealed the characteristics of the host CHO cell lines possessing high productivity and high quality, which are suitable for large-scale production.

At the first step in this study, I determined factors closely related to titer, which is a productivity indicator, and the area percentage of high molecular weight species [HMWS(%) as determined by size exclusion chromatography (SEC) analysis], which is equivalent to aggregate content and is used as a quality indicator, to characterize cells that have high productivity and low aggregates

contents. Twenty-eight stable CHO cell lines that produce trastuzumab (trade names Herclon, Herceptin) were generated, and their properties were analyzed, such as titer, HMWS(%) in culture media. To understand the relationship between various factors and titer/HMWS(%), I performed stepwise multiple linear regression analyses. I found that high titer was associated to high specific growth rate (μ), high specific production rate (Q_p), and low intracellular heavy chain (HC) protein content. Thus, the cell lines that exhibit high intracellular HC content due to their difficulties in the assembling/folding process in the endoplasmic reticulum (ER) are considered to exhibit decreased titer, and the HC protein accumulation is thought to induce unfolded protein response (UPR), which is unfavorable for the cells. On the other hand, low HMWS(%) was associated to a low PDI mRNA level, high LMWS(%), high Q_p, high intracellular LC protein content, and high μ. In addition, it was considered that the partially misfolded antibody molecules may cause aggregates in the culture medium or in the cells. Taken together, the presented results suggest that correct and efficient assembling and/or folding of an antibody molecule in the ER are important for high titer and low aggregate contents.

At the second step in this study, to identify the characteristics of cell lines that produce mAb with qualities suitable for large-scale production, I investigated whether the characteristics of antibody samples and cells differed between the two cell lines with contrasting productivities and aggregate contents. Cell line A (high titer and high quality) and cell line B (low titer and low quality) were selected from the 28 cell lines, and each cell line was cultured three times. The comparison of cell behavior and antibody samples between the two cell lines by using various analytical methods, such as SEC and electrophoresis revealed various differences (cell growth, the contents of noncovalent aggregates, accumulation of HC dimers/monomer, and proportion of defucosylated oligosaccharides). I attribute the higher aggregate content and lower titer in cell line B to the lower production levels of LC and more extensive subsequent accumulation of HC

dimers/monomers in cell line B. The major mechanisms of aggregate formation were also different between the two cell lines. The aggregates from cell line A were predominantly formed by covalent interaction, whereas those from cell line B were predominantly formed by hydrophobic interactions.

At the third step in this study, I investigated the influence of cell's metabolic states on mAb productivity/quality using metabolomic analyses. It was previously observed that the difference between cell line A and B in the productivity (titer) was attributed mainly to differences in the number of total cells (cell line A > cell line B). With respect to the qualities of mAbs secreted from the two cell lines, it was observed that there was a cell-type difference in the proportions of defucosylated oligosaccharides (cell line A < cell line B), which are related to the antibody-dependent cell cytotoxicity activity of mAbs. In addition, in the second step, I observed different proportions of covalent aggregates (cell line A > cell line B) although the total aggregate content was higher for cell line B than for cell line A. I investigated whether these differences were associated with the cell's metabolic state. My results suggest that the high mAb titer of cell line A is not accounted for by the lactate metabolism shift, although lactate metabolism shift is useful for selection of cell lines with high productivity. The differences in cell proliferation between high and low antibody-producing cell lines can be accounted for by the levels of tricarboxylic acid cycle intermediates. The difference in proportions of fucosylated oligosaccharides may be explained by the distinct levels and localization of fucosyltransferase rather than differences in the intracellular pool of GDP-fucose. Oxidative stress is likely involved in the difference in proportions of covalent aggregates, and the difference in oxidative stress between cell lines may be associated with mitochondrial oxidative activity.

In this study, various differences between monoclonal antibody-expressing cell lines with high productivity/high quality and with low productivity/low quality were revealed by approaches from

fermentation engineering, biophysical chemistry, and metabolomic analyses. The present findings will be useful not only for the selection/creation of cell lines with high productivity/high quality but also for improving manufacturing processes of mAbs.