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Elevation of gamma-glutamyltransferase activity in 293 HEK cells constitutively expressing antisense glutaminase mRNA

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Abstract

Previous studies have shown that the use of dynamic nutrient feeding to maintain glutamine at low levels in fed-batch cultures reduced the overflow of glutamine metabolism. This strategy resulted in the shift of metabolism towards an energetically more efficient state signified by reduced lactate and ammonia production and thus achieving a higher cell density for enhanced productivity. In an effort to mimic the metabolic changes effected by this fed-batch strategy at the molecular level, 293 HEK cells were engineered via stable transfection with an antisense fragment of the rat phosphate-dependent glutaminase (PDG) gene. PDG is localized in the mitochondria and catalyzes the deamination of glutamine to glutamate with the release of ammonia. Stable single cell clones were isolated from the transfected populations. Characterization of these transfectants revealed indications of an altered glutamine metabolism affected by the antisense strategy. Contrary to our expectations, glutamine consumption and ammonia production in the antisense cells did not deviate significantly from that of untransfected cells. Glutamate was also observed to accumulate to high level extracellularly, as opposed to a consumption pattern normally observed in non-transfected cells. Subsequent analyses show that gamma-glutamyltransferase (γ-GT) may be a significant pathway that resulted in the formation of glutamate and ammonia from glutamine catabolism extracellularly. γ-GT has been widely investigated in renal glutamine metabolism, but has rarely been implicated in cultured cell metabolism. This study highlights the importance of this alternative glutamine metabolism pathway in cell culture.

Keywords: 293 HEK; Cell culture; Glutaminolysis; Phosphate activated glutaminase (PAG); Gamma-glutamyltransferase; Antisense

1. Introduction

Highly active glucose and glutamine metabolism is widely reported in mammalian cell cultures, and it induces an unnecessarily high uptake of these nutrients, resulting in the production of inhibitory levels of waste metabolites like lactate and ammonia. The accumulation of inhibitory metabolites has been known to pose a limitation on the maximum attainable cell and product yields in mammalian cell batch cultures (Glacken, 1988; Hassell et al., 1991).

Dynamic nutrient feeding can be used to maintain glucose, glutamine or both at low levels in fed-batch cultures, and thus minimizes the production of lactate and ammonia. The strategy has been shown to enable the achievement of high cell density and productivity in hybridoma, BHK, and 293 HEK cultures (Zhou et al., 1995; Cruz et al., 2000; Siegwart et al., 1999; Lee et al., 2003). Although there might be concerns that limiting glucose and/or glutamine can affect product glycosylation in fed-batch cultures, as both nutrients play a major role in forming the precursors of glycan structures, it has been reported that optimizing set-point nutrient levels could prevent the loss of product glycosylation (Wong et al., 2004).

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Genetic manipulation of the metabolic pathways involving ammonia and lactate presents an alternative approach to regulate the accumulation of lactate and ammonia. Stable cell lines with reduced ammonia and/or lactate formation have been reported by overexpressing glutamine synthetase (Bebbington et al., 1992) and pyruvate carboxylase (Irani et al., 2002; Elias et al., 2003), and gene targeting of lactate dehydrogenase (Chen et al., 2001). A further approach to directly regulate glutamine metabolism is to modulate the expression of the chief enzyme responsible for its deamidation, glutaminase. Glutaminase exists in two isoforms, liver- and kidney-type, which are products of different but related genes. The two isozymes possess different kinetic properties and protein structure. The liver-type glutaminase is associated with glucogenesis and urea formation, and is detected only in cells with a functional urea cycle. The kidney-type glutaminase is expressed in all other organs that utilize large amount of glutamine, such as kidney, brain, intestine and fetal liver. Functional glutaminase activity is expressed on the cytosolic surface of the inner mitochondrial membrane and represents the dimeric form of the enzyme (Kvamme et al., 1991). It is responsible for the deamidation of glutamine to glutamate with the liberation of an ammonium molecule is activated by phosphate and glutamine (hence, phosphate activated glutaminase: PAG), and its inhibitors include glutamate and ammonia. Majority of the glutamine taken up by cells in culture undergo the PAG pathway for generation of energy, and only a small amount of glutamine is used for biosynthetic purposes.

The motivation of the current work is to limit glutamine uptake by directly modulating glutamine metabolism via the suppression of PAG. The generation of a 293 HEK cell line that permanently expresses reduced level of PAG might enable optimal glutamine regulation without complete elimination of its supplementation, which may affect glycosylation. The strategy may also reduce lactate and ammonia formation, as observed in glutamine controlled fed-batch strategy (Lee et al., 2003).

We have selected to utilize antisense technology to affect the permanent reduction of PAG as it has been shown to be a successful tool for blocking metabolic pathways by targeting fucosyltransferase VI, sialidase and caspase 3 in the studies by Prati et al. (1998), Ferrari et al. (1998), and Kim and Lee (2002), respectively. The antisense 293 HEK cell lines generated were compared to the parental cells in terms of growth, metabolism, and glutaminase expression. Glutamine metabolism was identifiably altered as a result of the attenuated PAG pathway. However, glutamine uptake and ammonia production were unchanged and extracellular glutamate accumulation was significantly high in the antisense cell lines. Based on these results, we deduced that an alternate glutamine catabolic pathway, via the enzyme γ-glutamyltransferase (γ-GT), might have played a significant role in 293 HEK cell glutamine metabolism. This will be demonstrated by the increase in γ-GT activity in the antisense cell lines.

2. Materials and methods

2.1. Cell culture

The anchorage-dependent 293 Human Embryonic Kidney (HEK) cell line was purchased from American Type Culture Collection (ATCC CRL-1573). The cell line was grown in DMEM medium (Invitrogen) supplemented with 10% (v/v) fetal calf serum (Hyclone). Cells were also adapted to grow in suspensions in serum-free medium, 293 SFM II (Invitrogen) supplemented with 4 mM glutamine (Sigma Aldrich). Anchorage dependent cells were subcultured every 3 days in tissue culture flasks. Suspension cells were subcultured every 3–4 days in shaker flasks. Both types of cells were grown at 37 °C with 8% CO_2 atmosphere.

2.2. Construction of antisense glutaminase plasmids

The pGA104 recombinant plasmid containing the rat kidney phosphate-dependent glutaminase (PDG) cDNA (Genbank Accession number: M65150) was kindly provided by Dr. Norman P. Curthoys (Colorado State University). Three fragments were obtained after restriction with EcoRI and HindIII. The fragment sizes were 1.6, 1.1 and 0.28 kb. The 3′-end sequences, 1.6 and 0.28 kb, were subcloned in their antisense orientations into the pcDNA3.1 vector containing a neomycin resistance gene (Invitrogen). The 3′-end sequences were selected because of the high sequence similarity found amongst cDNAs coding for C-terminal ends of known glutaminases (Lobo et al., 2000). The sequences selected for antisense targeting were specific to kidney glutaminase and do not show homology to other genes, as verified by NCBI BLAST. The resulting constructions were designated pPDG1.6AS and pPDG0.28AS.

2.3. Generation of antisense glutaminase stable cell lines

293 HEK cells were transfected using Lipofectamine^{TM} 2000 reagent (Invitrogen), as per manufacturer's instructions, to obtain the stable cell lines 293-1.6AS (1.6 kb antisense fragment) and 293-0.28AS (0.28 kb antisense fragment). G418 (Sigma-Aldrich) was added at a concentration of 700 μg/ml and drug resistant cells were collected after 2–3 weeks for single cell cloning, in which cells were diluted, and seeded in 96-well plates at one cell per well. Wells that contained more than 1 cell were marked and excluded from further investigation.