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Full paper

Differentiated HASTR/ci35 cells: A promising *in vitro* human astrocyte model for facilitating CNS drug development studies

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ABSTRACT

Astrocytes have shown longstanding promise as therapeutic targets for various central nervous system diseases. To facilitate drug development targeting astrocytes, we have recently developed a new conditionally immortalized human astrocyte cell line, termed HASTR/ci35 cells. In this study, in order to further increase their chances to contribute to various astrocyte studies, we report on the development of a culture method that improves HASTR/ci35 cell differentiation status and provide several proofs related to their astrocyte characteristics. The culture method is based on the simultaneous elimination of serum effects and immortalization signals. The results of qPCR showed that the culture method significantly enhanced several astrocyte marker gene expression levels. Using the differentiated HASTR/ci35, we examined their response profiles to nucleotide treatment and inflammatory stimuli, along with their membrane fatty acid composition. Consequently, we found that they responded to ADP or UTP treatment with a transient increase of intracellular Ca^{2+} concentration, and that they could show reactive response to interleukin-1β treatments. Furthermore, the membrane phospholipids of the cells were enriched with polyunsaturated fatty acids. To summarize, as a unique human astrocyte model carrying the capability of a differentiation induction properties, HASTR/ci35 cells are expected to contribute substantially to astrocyte-oriented drug development studies.

1. Introduction

Astrocytes are a type of glial cells abundantly found in mammalian brains, where they play pivotal roles in various physiological central nervous system (CNS) functions. For example, astrocytes can respond to extracellular ATP in order to induce transient elevation of intracellular Ca^{2+} concentration through two families of purinergic (P2X and P2Y) receptors, which consequently triggers a variety of reactions, including gliotransmitter releases, and thus helps precisely regulate synaptic activity.[1–3] In addition, astrocytes are critically involved in the synaptic clearance of various neurotransmitters, neuron energy supply, blood flow regulation, as well as the maintenance of blood–brain barrier integrity.[4,5]

Furthermore, it has long been known that astrocytes can respond to CNS injury and diseases, in association with drastic morphological and functional alterations, as exemplified by hypertrophy, upregulation of glial fibrillary acidic protein (GFAP), and the secretion of a variety of trophic factors. When in this state, they are referred to as reactive astrocytes. It has been shown that they exhibit both detrimental and favorable aspects unevenly balanced in a disease context- and timing-dependent manner.

Due to their above-mentioned importance in normal as well as diseased CNS functions, astrocytes have shown longstanding

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promise as various therapeutic targets. In such drug development, *in vitro* astrocyte models can provide tools that allow not only for drug screening, but also for mechanistic characterization of their mode-of-action (for example, Makitani et al.[6]), in which astrocytes of human origin are likely to be preferable to rodent cells due to the significant morphological and functional species differences that have been reported to date.[7]

Meanwhile, we have recently reported on the establishment of a new conditionally immortalized human astrocyte cell line, termed HASTR/ci35.[8] Owing to the functions of the temperature-sensitive simian virus 40 large T antigen (tsSV40T) and the human telomerase catalytic subunit (hTERT), HASTR/ci35 cells can proliferate extensively. This is in clear contrast to primary human astrocytes (prHASTR), which must be used sparingly due to their finite proliferation capacity. In addition, HASTR/ci35 cells show several astrocytic characteristics.[8] However, HASTR/ci35 cells are morphologically different from prHASTR, and their astrocytic marker mRNA expression levels appear to be lower than those of prHASTR, thereby pointing to need for further improvements to their differentiation status.

One of the ways to take on this challenge is the elimination of growth signaling, which often overwhelmingly counteracts cell differentiation properties. In HASTR/ci35 cells cultured with the previously described method, there are two growth signaling sources: one is fetal bovine serum (FBS) in the medium, and the other is tsSV40T function, the latter of which confers a conditionally immortalized nature on HASTR/ci35 cells. More specifically, while tsSV40T is stably expressed in 33 °C culture used to promote cell proliferation primarily through inhibiting p53 and retinoblastoma protein (pRb) functions, the protein is rapidly broken down when the culture temperature goes above 37 °C.

Therefore, we have developed a culture method that facilitates HASTR/ci35 cell differentiation. Furthermore, by utilizing the differentiated HASTR/ci35 cells, we have provided additional evidence for their astrocyte functions, along with intriguing insights into their unique membrane fatty acid profile.

2. Materials and methods

2.1. HASTR/ci35 cell culture and differentiation

HASTR/ci35 cells (passages 30–50) were routinely grown at 33 °C with 5% CO_2/95% air in Dulbecco's modified eagle medium (DMEM) (Thermo Fisher Scientific, Waltham, MA) supplemented with GlutaMAX-I, 1% N2 supplement, 10% (v/v) FBS, penicillin-streptomycin and 4 μg/mL blasticidin S (hereafter referred to as the complete astrocytes medium (CAM)). The culture condition with CAM at 33 °C is hereafter indicated as [33 °C/10%FBS]. The culture dishes were coated with collagen type I.

For HASTR/ci35 cell differentiation, CAM without FBS was used, and the culture temperature was set at 37 °C [37 °C/FBS-free]. The culture condition using CAM without FBS at 33 °C [33 °C/FBS-free] and that with FBS at 37 °C [37 °C/10%FBS] were also employed for a comparison. Gene expression or functional analyses were conducted at 24 h unless otherwise stated.

2.2. Cell labeling

For HASTR/ci35 cell labeling, the cells were suspended in pre-warmed CellTracker Green CMFDA (5-chloromethylfluorescein diacetate, Thermo Fisher Scientific) solution for 45 min at 37 °C. The labeled cells were observed by a confocal fluorescence microscopy.

2.3. Cell proliferation analysis

One day after seeding at 5.0×10^4 cells/mL (day 0) with [33 °C/10%FBS], three different culture conditions [33 °C/10%FBS], [33 °C/FBS-free] and [37 °C/FBS-free] were applied. The cell numbers were determined by direct cell counting at day 3, 5 and 9.

2.4. Immunocytochemistry

The cells were grown with [33 °C/10%FBS] and then switched to [37 °C/FBS-free]. The cells were fixed with 4% paraformaldehyde (PFA) and permeabilized with 0.1% Triton-X100, after which they were blocked with 3% bovine serum albumin (BSA). The primary and secondary antibodies used are shown in Table S1. For nuclear counter-staining, 4′,6-diamidino-2-phenylindole (DAPI) was used.

2.5. Total RNA extraction, cDNA synthesis and qPCR

Total RNA extraction and cDNA synthesis were performed as described previously.[9] Quantitative PCR (qPCR) was conducted using the primers listed in Table S2. The target mRNAs are indicated in the result section. Data were obtained using the delta-delta-CT method, where glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels were used as a control.

2.6. Ca^{2+} imaging

HASTR/ci35 cells cultured with [37 °C/FBS-free] were prepared. The cells were incubated with 2 μM Fluo-4 AM (Dojindo, Kumamoto, Japan) according to the manufacturer's protocol. After the addition of 100 μM ADP containing either 1 μM MRS2179 (Cayman chemical, Ann Arbor, MI, USA) or its vehicle, dimethyl sulfoxide (DMSO), the time-dependent change of intracellular Ca^{2+} concentration was measured. The Ca^{2+} response was also examined using 100 μM UTP in the presence or absence of 10 μM AR-C118925XX (Tocris Bioscience, Bristol, UK). The fluorescence intensity at each time point was represented as $\Delta F/F_0$, where ΔF indicates $F\text{-}F_0$, F indicates the fluorescence intensity at each time point, and F_0 indicates the value obtained by averaging the fluorescence intensity obtained for four cycles before the addition of ADP or UTP.

2.7. Pro-inflammatory cytokines and lipopolysaccharide treatments

HASTR/ci35 cells cultured with [37 °C/FBS-free] were prepared and then treated with 50 ng/mL tumor necrosis factor-α (TNF-α) (Peprotech, Rocky hills, NJ), 10 ng/mL interleukin-1β (IL-1β) (Peprotech), 100 ng/mL lipopolysaccharide (LPS) (Sigma), or sterile water. For TNF-α or IL-1β treatment, the cells were further cultured for 96 hr, during which the medium was once replaced with a fresh medium containing either one of test reagents at 48 hr. For LPS treatment, the cells were incubated for 48 hr. Subsequently, gene expression analyses were performed as described in sections 2.4 and 2.5. For a comparison purpose, similar experiments were conducted using prHASTR and HASTR/ci35 cells cultured with [33 °C/10%FBS].

2.8. Lipidomic analysis

The cells were initially grown in three 100-mm culture dishes with [33 °C/10%FBS] for a week and then switched to [37 °C/FBS-free]. For comparison purposes, we used human colon carcinoma (HCT116), human embryonic kidney 293 (HEK293), and human hepatocarcinoma (HepG2) cells, all of which are widely used with

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