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Cell cycle regulation by glucosamine in human pulmonary epithelial cells

Kun-Han Chuang [a], Chih-Shen Lu [b], Yu Ru Kou [a], Yuh-Lin Wu [a,*]

[a] Department of Physiology, School of Medicine, National Yang-Ming University, No. 155, Sec. 2, Linong Street, Beitou District, Taipei 112, Taiwan
[b] Department of Neurosurgery, Cheng Hsin General Hospital, No. 45, Cheng Hsin Street, Beitou District, Taipei, Taiwan

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ABSTRACT

Airway epithelial cells play an important role against intruding pathogens. Glucosamine, a commonly used supplemental compound, has recently begun to be regarded as a potential anti-inflammatory molecule. This study aimed to uncover how glucosamine impacts on cellular proliferation in human alveolar epithelial cells (A549) and bronchial epithelial cells (HBECs). With trypan blue-exclusion assay, we observed that glucosamine (10, 20, 50 mM) caused a decrease in cell number at 24 and 48 h; with a flow cytometric analysis, we also noted an enhanced cell accumulation within the G_0/G_1 phase at 24 h and induction of late apoptosis at 24 and 48 h by glucosamine (10, 20, 50 mM) in A549 cells and HBECs. Examination of phosphorylation in retinoblastoma (Rb) protein, we found an inhibitory effect by glucosamine at 20 and 50 mM. Glucosamine at 50 mM was demonstrated to elevate both the mRNA and protein expression of p53 and heme oxygenase-1 (HO-1), but also caused a reduction in p21 protein expression. In addition, glucosamine attenuated p21 protein stability via the proteasomal proteolytic pathway, as well as inducing p21 nuclear accumulation. Altogether, our results suggest that a high dose of glucosamine may inhibit cell proliferation through apoptosis and disturb cell cycle progression with a halt at G_0/G_1 phase, and that this occurs, at least in part, by a reduction in Rb phosphorylation together with modulation of p21, p53 and HO-1 expression, and nuclear p21 accumulation.

1. Introduction

Airway epithelial cells are in direct contact with inhaled materials, including pollutants, allergens and microbes as well as other factors, and these factors are important to the development of inflammation in the respiratory system [1]. Inflammation may initiate genetic and epigenetic changes that lead to regulation of a variety of cellular functions, such as proliferation, survival and apoptosis [2]. In fact, inducible inflammatory mediators have been demonstrated to play significant roles in cell proliferation and cell cycle progression via the modulation of anti-apoptotic proteins and cell cycle regulators; these changes involve multiple signaling pathways in both human lung adenocarcinoma and bronchial epithelial cells [3].

Glucosamine is a natural amino monosaccharide involved in the formation of cartilage and it has been widely used to treat human osteoarthritis-related symptoms [4]. Glucosamine has been reported to have anti-inflammatory and immune-regulatory functions that are related to a reduced expression of inflammatory mediators [5]. At the same time, glucosamine also has been demonstrated to induce cytotoxicity in pancreatic beta cells [6]. In addition, recent studies have noted that a suppression of proliferation occurs when various cancer cell types are treated with glucosamine [7,8]. However, the effects of glucosamine in cell proliferation of airway epithelial cells and the molecular mechanisms by which glucosamine acts remain unknown.

The cell cycle is controlled by several cell cycle regulators, including various kinds of cyclin-dependent kinase inhibitors (CDKIs), such as p21 and p27 being well-recognized to inhibit the functions of most complexes of cyclins and cyclin-dependent kinases (CDKs), those are required for cell cycle progression [9,10]. In addition, induction of heme oxygenase isoform-1 (HO-1), a major kind of heme-degrading enzyme, has also been shown to be associated with the inhibition of the proliferation in several cell types, including human pulmonary epithelial cells [11–13].

Based on our previous studies, it is known that glucosamine is able to effectively suppress the LPS-induced expression and subsequent secretion of inflammatory mediators in primary human bronchial epithelial cells (HBECs) [14]. However, the impact of glucosamine on the cell growth, cell cycle and apoptosis of human primary cells or human airway epithelial cells has not been characterized. In this study, the aim was to investigate the potential

* Corresponding author. Tel.: +886 2 2826 7081; fax: +886 2 2826 4049.
E-mail address: ylwu@ym.edu.tw (Y.-L. Wu).

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impacts of glucosamine on cell proliferation, cell cycle progression as well as cell apoptosis in human respiratory epithelial cells at the molecular level and to determine how this occurs.

2. Materials and methods

2.1. Chemicals and reagents

Fetal bovine serum (FBS) was obtained from HyClone (Logan, UT, USA). Glucosamine hydrochloride, MG132 and chloroquine were from Sigma (St. Louis, MO, USA). Reverse transcriptase and Taq polymerase were from Promega (Madison, WI, USA). Antibodies against p21, histone H1, Rb and p-Rb were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against p27 was from Cell Signaling Technology (Danvers, MA, USA). Antibody against p53 was from NeoMarkers (Fremont, CA, USA). Antibody against HO-1 was purchased from Enzo Life Sciences International Inc. (Plymouth Meeting, PA, USA). Donkey anti-rabbit IgG secondary antibody was purchased from Amersham Life Science Inc. (Arlington Heights, Illinois, USA). Unless otherwise specified, all other chemicals and reagents used in this project were from Sigma.

2.2. Cell culture

A human alveolar epithelial cell line (A549) was from the American Type Culture Collection (Rockville, MD, USA) and used as a model of human alveolar epithelial cells. Primary human bronchial epithelial cells (HBECs) were from Cell Application Inc. (San Diego, CA, USA). Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere in F12K medium (Sigma) containing 10% fetal bovine serum and 1% penicillin and streptomycin. Cells were plated on the previous day and grown for 16–18 h prior to various treatments, which were carried out on the following day.

2.3. Determination of cell viability by trypan blue exclusion assay

A549 cells and HBECs were seeded in 6-well plates at a density of 8×10^5 cells per well overnight. After serum-starvation for 24 h, cells were treated with different concentrations of glucosamine for 24 or 48 h. Cells were then trypsinized and mixed (1:1 vol/vol) with 0.4% trypan blue solution for staining and the suspension was added to a hemocytometer. Viable (unstained) and nonviable (blue) cells from each well were counted over eight microscopic fields.

2.4. Cell cycle and apoptosis analyses

To determine the cell cycle distribution profiles, cells were seeded at 8×10^5 cells per well in 6-well plates overnight. After serum-starvation, cells were exposed to different concentrations of glucosamine for 24 or 48 h. The cells were then trypsinized, harvested using cold PBS, fixed in 70% ethanol and incubated with fluoro-chrome DNA staining solution containing 50 μg/ml propidium iodide (PI) and 100 μg/ml RNAse A for 40 min at 37 °C in the dark. To determine the apoptosis rate, an annexin V-fluorescein isothiocyanate (annexin V-FITC) and PI apoptosis detection kit (Sigma) was used to determine the early and late apoptotic activities according to the manufacturer's instructions. Overnight plated cells in 6-well plates were treated with different concentrations of glucosamine for 24 or 48 h, and the cells were harvested and resuspended in 100 μl of binding buffer. A total of 10 μl of annexin V-FITC and 10 μl of PI were added and the mixture was incubated for 30 min in the dark. Finally, 400 μl of binding buffer was added to the cells and the mixture was analyzed. The distribution of cells across the cell cycle and apoptotic analysis were determined by flow cytometry (Becton Dickinson FACSCalibur, Franklin Lakes, NJ, USA). Data were processed using CXP FC500 software (Beckman Coulter, CA, USA).

Table 1
Primers used and the sizes of PCR products.

Gene	Sequence	Size (bp)
p21	Forward-5′ GCC GCG ACT GTG ATG CGC TAA TG -3′ Reverse-5′ CCG GCG TTT GGA GTG GTA GA -3′	377
p27	Forward-5′ AGA GGC GAG CCA GCG CAA -3′ Reverse-5′ CTG CTC CAC AGA ACC GGC A -3′	400
p53	Forward-5′ CGG TTT CCG TCT GGG CTT CT -3′ Reverse-5′ GCA CCT CAA AGC TGT TCC GTC CC -3′	497
HO-1	Forward-5′ CAG GCA GAG AAT GCT GAG TTC -3′ Reverse-5′ GAT GTT GAG CAG GAA CGC AGT -3′	555
β-actin	Forward-5′ GGC ACC ACA CCT TCT ACA AT -3′ Reverse-5′ CGT CAT ACT CCT GCT TGC TG -3′	833

2.5. Western blotting

Overnight plated cells were treated with different concentrations of glucosamine for 24 or 48 h and harvested with lysis buffer (50 mM Tris, 5 mM EDTA, 300 mM NaCl, 1% Triton X-100, 1 mM PMSF, 100 ng/ml Aprotinin and 100 ng/ml Leupeptin-Hemisulfate). The harvested cell lysates were centrifuged at 12,000 rpm for 15 min at 4 °C and the supernatant was collected as the total cellular protein sample. Nuclear proteins were obtained by Nonidet P-40 lysis buffer (10 mm HEPES, pH 7.4, 10 mm KCl, 1.5 mm $MgCl_2$, 0.02% sodium azide, 0.5% Nonidet P-40, 100 mM PMSF, 0.1% Aprotinin and 0.1% Leupeptin-Hemisulfat), followed by

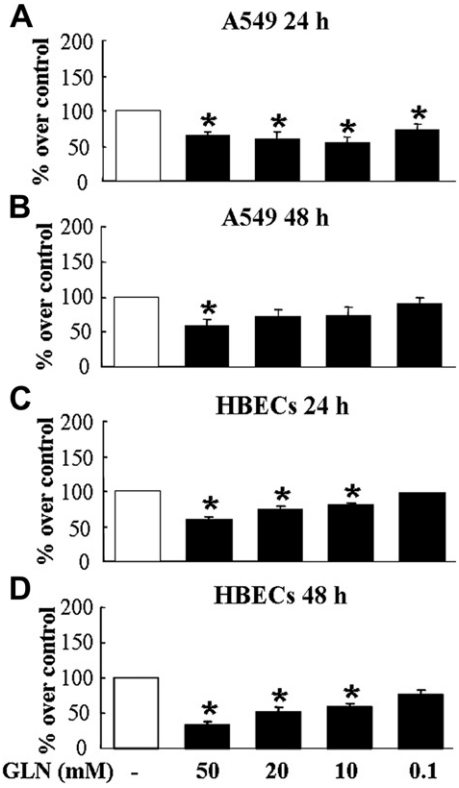

Fig. 1. Inhibition of cell proliferation by glucosamine in A549 cells and HBECs. Cells were serum starved for 24 h, followed by treatment with various doses of glucosamine for 24 h (A, C) or 48 h (B, D) to count alive cell numbers by trypan blue-exclusion assay. Each value represents means ± S.E.M. from four independent experiments $^*p < 0.05$ compared with the control group.