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Homocysteine influences the human keratocytes cell cycle

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ABSTRACT

BACKGROUND: Homocysteine (Hcy), a metabolic intermediate, is a sulfur-containing amino acid not present in the structure of proteins. It has been shown that high Hcy levels via oxidative stress, induced inflammation, and vessel dysfunction may affect the functioning of tissues, including the structures that form the eye, among others keratocytes. The visual disturbance caused by high levels of Hcy may also be associated with disturbed cell proliferation resulting from the effect of this amino acid on the cell cycle. The goal was to analyse the influence of Hcy on the keratocytes and to find out in which phase of the cell cycle its course is disturbed by this amino acid.

MATERIALS AND METHODS: A normal human keratocytes (HK) cell line was used in the study. May-Grünwald-Giemsa (MGG) staining for morphology visualization and cytometric cell cycle analysis were performed.

RESULTS: Hcy does not affect the G1 phase of the cycle, while it regulates the S and G2 phases. Changes in the amount of the sub-G1 population indicative of a pro-apoptotic effect of Hcy on keratocytes were detected. The form of the Hcy administered (L stereoisomer or DL racemic mixture) and the amino acid concentration were also important.

CONCLUSIONS: Homocysteine influences the keratocyte cell cycle. The change occurs at the stage of the G1-S transition, which suggests a decreasing level of cells in the S phase and an increasing level in the G2 phase. The long-term influence of Hcy on keratocytes may affect keratocytes proliferation and possible cornea regeneration.

KEY WORDS: homocysteine; human keratocytes; cell cycle

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INTRODUCTION

Homocysteine (Hcy) is an endogenous, sulfur-containing amino acid that connects the folate cycle with methionine. This amino acid is not a part of proteins and is not supplied to the body in food. In cells, it is formed from methionine in the processes of demethylation and hydrolysis. In turn, homocysteine metabolism occurs by transsulfuration and re-methylation [1]. These processes require the presence of vitamin B6 or B12, functioning as coenzymes. Disruption of these processes may lead to increased Hcy concentration in plasma to values exceeding the physiological concentration (10 μM) and, consequently, the appearance of ho-

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mocysteinemia or hyperhomocysteinemia symptoms [2]. Persistently elevated Hcy levels in body fluids may be a risk factor for the development or progression of changes in eye tissues affecting the visual process. It has been indicated that an increase and a sustained high concentration of Hcy in the aqueous humor of the anterior chamber or in tears may be a serious and potential cause of eyesight problems and even blindness [3]. It has also been postulated that Hcy may play a role in the pathogenesis of keratoconus (KC) manifested by common ectatic corneal dystrophy associated with progressive stromal thinning and loss of structural integrity [4].

By activating oxidative stress and stimulating inflammatory processes, Hcy metabolites contribute to degenerative changes in the vascular system, modification of the extracellular matrix structure, and abnormal stimulation of cell proliferation related to, e.g., endothelial dysfunction [5]. There are studies showing that Hcy can influence the course of particular phases of the cell cycle. The cell cycle progress is regulated by the activity of the cyclin-cyclin-dependent kinases complexes (cyclin-cdks) [6]. They regulate this highly conservative process, which enables genetic duplication and cell division. The cdk-cyclin complexes guide the cell through G0/G1 (gap 1), S (DNA synthesis), G2 (gap 2), and M (mitosis) phases. Particular attention should be paid to the G1 and G2 phases as important regulators of cell cycle checkpoints. In turn, the restriction point between G1 and S phases determines whether the cell will enter the S phase of the division cycle or quit this process by entering the resting state G0 [7].

Cyclin-dependent kinases are activated and positively regulated after their binding to cyclins. It was shown that G1-S cell cycle phase transition is regulated by cyclin E-cdk2 or cyclin D-cdk4/6. The cyclin A-cdk2 complex terminates the S phase, while the cyclin A-cdk1 complex enables cells to enter the M stage [7, 8]. On the other hand, cyclin-cdks themselves are also negatively regulated by cdk inhibitors that promote cell cycle arrest [9]. Studies on neuronal or endothelial cells have shown that also HHcy can affect the functioning of both cyclins and cyclin-dependent kinases and, thus, cell cycle inhibition.

Human keratocytes (corneal fibroblasts) remain in the quiescent state and only rarely undergo cell division under normal conditions. They reside in the corneal stroma as principal cells. They take part in maintaining cornea transparency, keeping corneal shape, and synthesizing or modifying its components. However, due to an accidental injury, surgical intervention, or inflammation, keratocytes become activated and phenotypically transformed into fibroblasts [10, 11]. As a result of some external stimuli influences, activated keratocytes (corneal fibroblasts) express particles or biologically active mediators (chemokines or adhesion molecules), which selectively (depending on the stimulus) recruit and activate inflammatory cells. This is to protect the eye surface and regenerate it. However, too much activity of these cells can lead to excessive corneal stroma degradation and even corneal ulceration [11].

In the proposed study, the cell cycle of corneal fibroblasts exposed *in vitro* to homocysteine at physiological concentrations and under conditions similar to hyperhomocysteinemia was performed. The aim of these analyzes was to show the influence of Hcy on the tested cells and to find out in which phase of the cell cycle its course is disturbed.

MATERIAL AND METHODS

Cell lines

A normal human keratocytes or corneal fibroblast cell line (HK) (Cat. #6520) (ScienCell Research Laboratories, Inc. Carlsbad, CA, United States) was used. The cells were cultured as monolayers in 75 cm^2 culture flasks (NuncTM, Roskilde, Denmark), coated with Poly-L-Lysine (PLL) (ScienCell Research Laboratories) at 10 mg/ml concentration (about 130 mg/cm^2). The cell lines were maintained in fibroblast medium (FM) with fibroblast growth supplement (FGS) (ScienCell Research Laboratories) and serum-free medium (FBS) (ScienCell Research Laboratories). The sterile liquid medium was supplemented with antibiotics: a 1% penicillin/streptomycin (P/S) solution (ScienCell Research Laboratories) at 37°C in a humidified atmosphere with 5% CO_2.

Experimental design

Cells were cultured in 6-well plates (Nunc^{TM}) at 1×10^5 cells/mL initial density. After 24 hours of inoculation, the medium was replaced with a new one containing Homocysteine (Hcy) stereoisomer L or a racemic mixture DL at a concentration range of 1 μM–1 mM). The culture was conducted for

further 24 hours. After this time, the cells were subjected to cytometric analysis.

May-Grünwald-Giemsa (MGG) staining

The cells were incubated in 24-well plates in 1 ml of culture medium supplemented with homocysteine. After 24 hours of incubation (37°C in a humidified 5% CO_2/95% air), the medium was discarded, and the cell cultures were rinsed with FM medium and stained with May-Grünwald (MG) stain for 5 min followed by staining for another 5 min in MG diluted in an equal quantity of water. The MG was removed, and a Giemsa reagent (diluted 1:20 in water) was added to the cells, which were next incubated at room temperature for 15 minutes. Thereafter, the cells were rinsed three times with water, dried, and subjected to microscopic observations (Olympus, BX51; Olympus).

Cytometric analysis of the cell cycle

To examine the influence of homocysteine L or DL concentrations on the distribution of cells in the cell cycle phases, the HK cells, after incubation with Hcy floating and adherent cells, were harvested, centrifuged (3000 rpm/5 min), rinsed in PBS w/o Ca^{2+} and Mg^{2+} ions, once again centrifuged, and fixed in 70% ethanol. The control was constituted by the untreated cells. The samples were stored for 1 week at –20°C. After this time, the samples were subjected to further steps of the PI staining procedure (PI/RNase Staining Buffer, BD PharmingenTM, BD Biosciences, San Jose, CA, United States). The PI fluorescence intensity was measured using FACS Calibur (BD PharmingenTM), and the obtained data were analyzed using Cell Quest Pro Version 6.0. for the Macintosh operating system (BD PharmingenTM). The results were calculated as a percent of cells in the respective cell cycle phases (sub-G1, G0/G1, S, and G2) among all the analysed cells. In total, 10.000 events were measured per sample.

Statistical analysis

The results are presented as ± standard deviation (SD) means of three independent experiments (n = 3). The data were analyzed using a one-way analysis of variance ANOVA followed by Dunnett's multiple comparison posthoc test. Differences of $p \leq 0.05$ were considered significant. Only results with a significance of $p \leq 0.05$ were reported.

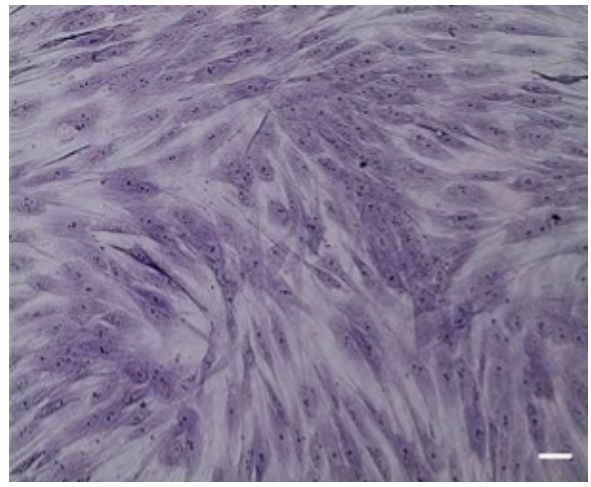

FIGURE 1. Human keratocytes (HK) cells stained with the May Grünwald-Giemsa (MGG) method. Morphology of the cells. Bar = 20 μm. Magnification 100×

RESULTS

The studies carried out with the Alamar blue and the Neutral Red uptake methods did not show a decrease in the viability of HK cells after 24 h incubation with homocysteine L and the racemic DL form. Only 2% fluctuations were observed as compared to the control (data not shown).

Similarly, no morphological changes in the cells were found in the culture when the cells were stained with the May Grünwald-Giemsa (MGG) method. Figure 1 shows the cell morphology following MGG staining.

Cytometric analysis of the cell cycle

To explore the influence of the selected homocysteine concentrations (1, 10, 100 µM, and 1 mM) on the distribution of cell cycle phases after 24 h incubation with HK cell line was analyzed by flow cytometry (Fig. 2).

The racemic mixture of homocysteine induces fewer changes in the number of cells in particular phases of the cell cycle than the L form compared to the cell cycle phases of the control. All results are summarized in Table 1.

Homocysteine L at a concentration of 1 mM increased the number of cells in the sub-G1 phase characteristic for apoptotic cells. It was 2.95%, which was an increase of 0.8% (a value greater than 1.37 times) in relation to the control. Moreover, homocysteine L at this concentration also decreased by 3.3% the number of cells in the S phase of the interphase cycle.

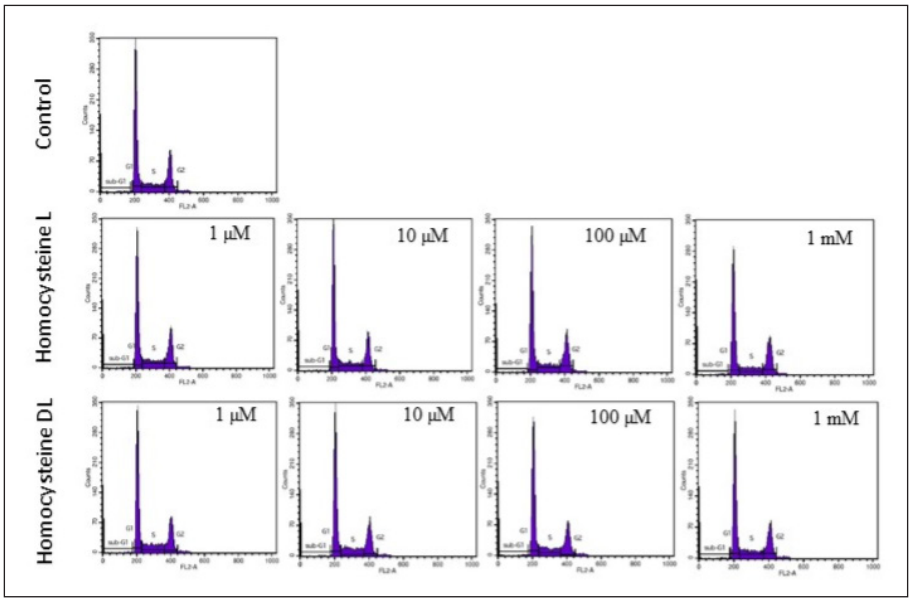

FIGURE 2. Effect of homocysteine L and DL on cell cycle distribution in HK cell culture analyzed by flow cytometry. Cells were treated with 1, 10, 100 μM, and 1 mM of Hcys for 24 hours. The cells were thereafter stained with propidium iodide and analyzed by flow cytometry. Representative DNA histograms for the tested cell line

Table 1. Statistical analysis of the percentages of HK cells in the sub-G1, G1, S, and G2 phases after 24 hours of incubation with different homocysteine L or DL concentrations

Cell cycle phase	Control	1 μM	10 μM	100 μM	1 mM
Homocysteine L					
Sub-G1	2.15 ± 0.10	1.98 ± 0.14	2.08 ± 0.27	1.99 ± 0.08	2.95 ± 0.08*
G1	53.83 ± 0.96	52.12 ± 0.93	53.31 ± 0.26	53.21 ± 0.46	52.05 ± 0.81
S	19.75 ± 0.80	19.45 ± 0.62	19.57 ± 0.84	19.18 ± 1.08	16.45 ± 0.98*
G2	23.68 ± 0.25	26.03 ± 0.43*	24.58 ± 0.65	25.24 ± 1.42	28.74 ± 0.63*
Homocysteine DL					
Sub-G1	2.15 ± 0.10	1.83 ± 0.05*	1.96 ± 0.39	2.09 ± 0.12	2.06 ± 0.09
G1	53.83 ± 0.96	53.23 ± 1.37	54.21 ± 1.55	53.75 ± 0.85	53.35 ± 0.62
S	19.75 ± 0.30	20.14 ± 0.58	18.31 ± 1.18	18.94 ± 0.52	18.43 ± 0.47*
G2	23.68 ± 0.25	24.50 ± 0.53	24.46 ± 0.95	24.59 ± 0.86	25.17 ± 0.45*

The addition of homocysteine L form to the cell culture increased the number of cells in the G2 phase compared to the control. The most substantial increase in the number of cells in the G2 phase was observed after incubating cells with homocysteine L at a concentration of 1 mM. There was an increase in G2 fraction by 5.06%.

On the other hand, the DL form of homocysteine did not significantly affect the changes in the number of cells in the sub-G1, G1, and S phases compared to the control. The racemic mixture at a concentration of 1 mM only increased the number of cells in the G2 phase. It was 1.49% higher than the one observed in the control.

DISCUSSION

The presented studies were performed to investigate how homocysteine affects the cell cycle of keratocytes. We have shown that Hcy does not affect the G1 phase of the cycle but regulates the S and G2 phases. Furthermore, we found changes in the amount of the sub-G1 population indicative of a pro-apoptotic effect of Hcy on keratocytes.

The form of the Hcy administered (L stereoisomer or DL racemic mixture) and the amino acid concentration were also important. Increasing Hcy levels can adversely affect the functioning of organs and tissues in the body, including eye tissues. It has been shown that the level of homocysteine in the retina is disturbed in people diagnosed with diabetic retinopathy, and the increasing level of Hcy in the retina can significantly damage its microvascularization. Moreover, excessive Hcy levels can damage the mitochondria of retinal cells and upset the DNA methylation status of enzymes responsible for transsulfuration and re-methylation of this amino acid [12]. Homocysteine also affects the degree of cell proliferation, which is essential in the case of activated keratocytes in the form of corneal fibroblasts. This is done by inhibiting cyclins or cyclin-dependent kinases (cdks) expression via a hypomethylation-related mechanism. This is especially important because the cell cycle progression is generally regulated by the activity of various cyclin-dependent kinases or their complexes with cyclins. Growth inhibition or the cytotoxic effect of excessive Hcy concentration is explained by the mechanism that may be involved is an endoplasmic reticulum (ER) stress. In turn, up-regulation of ER stress-related genes can induce cell cycle inhibition and even cell apoptosis [9]. It may also be suggested, consistently with Bourckhardt et al., that Hcy activity is not necessarily related to the classical P53-mediated cell cycle regulatory pathway. The changes in P53 levels may be involved not necessarily in regulating cell proliferation and DNA damage repair but in a standard program involving pro-apoptotic activity [13]. However, this does not change the fact that Hcy is involved in regulating the cell cycle at the interphase stage, which is reflected at the level of tissue disorders. Hcy may induce cell cycle arrest in G1 phase via the PI3K/Akt/FOXO signaling pathway [14]. Ye and Blain showed that Hcy influenced cyclin D1, and cyclin-dependent kinases 4 and 2 that translocated to the nucleus. These cyclins and kinases influence the G1-S phase transition. Moreover, it was found that the proteins associated with the G1 phase of the cell cycle were rapidly activated by Hcy. It can therefore be concluded that the inhibition of G1 phase progression may play a role in the protection of cells against apoptosis caused by an excessive concentration of homocysteine. Moreover, in addition to the G1 transition, also entry into the S phase of the cell cycle may be a critical factor in Hcy-induced apoptosis [6]. It was also found that changes in the expression of cell cycle markers such as cyclin D1 or Ki67 may undergo significant changes in disease states such as keratoconous eyes. Hence, any changes in the cell cycle parameters affecting the reduction of some markers may be the basis for limiting the effects associated with corneal stroma abnormalities [15].

CONCLUSION

The analyzes reported in this paper indicate that homocysteine influences the keratocyte cell cycle. The change occurs at the stage of the G1-S transition, which suggests a decreasing level of cells in the S phase and an increasing level in the G2 phase. The consequence of Hcy's long-term influence on keratocytes may be their apoptosis and problems with the regeneration of the corneal damage.

Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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