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Microfluidic Chip as a Tool for Effective *In Vitro* Evaluation of Cyclophosphamide Prodrug Toxicity

N. V. Pulkova[1], A. N. Zyrina[2], N. A. Mnafki[3], and I. M. Kuznetsova[4]

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Most drugs are metabolized in the liver, which can lead to their activation or inactivation with a change in the parent compound pharmacology, as well as liver damage by active metabolites. Preclinical animal studies of drug safety do not always predict its effect on humans due to species specificity. Thus, for the rapid drug screening, and especially prodrugs, an *in vitro* system is required that allows predicting xenobiotic cytotoxicity with consideration of their metabolism in liver cells. The use of a microfluidic chip (BioClinicum) made it possible to cultivate a 2D culture of human HaCaT keratinocytes with spheroids of human hepatoma HepaRG cells. After incubation in a specially selected universal serum-free medium containing 3.8 mM cyclophosphamide, pronounced death of HaCaT cells was observed in comparison with culturing in the absence of liver cells.

Key Words: *serum-free medium; human keratinocytes of the HaCaT line; microfluidic chip; spheroids of the HepaRG human hepatoma cell line; cyclophosphamide*

In toxicological studies, a comprehensive risk assessment is based on the assumption that the effect observed in laboratory animals will also be manifested in humans, but this concept is justly criticized due to species specificity. In preclinical studies *in vivo*, the risk cannot be fully predicted; it can only be determined at the stage of clinical trials.

For evaluation of the cytotoxicity of the test substances in order to increase the informative value of studies, as well as to reduce the number of laboratory animals, alternative *in vitro* tests using normal or transformed human cell lines are currently being developed. In addition, in toxicological studies, it is necessary to take into account the biotransformation of drugs in liver cells, especially in case of prodrugs, because their metabolites can have pronounced toxic effects on both liver cells and cells of the target organ. The development of microfluidic technologies enables co-culturing of several types of cells (including liver cells) in a single circuit. A multi-well device continuous supply of nutrient medium and oxygen was developed for assessment of drug hepatotoxicity [11]. It allowed co-culturing of primary rat hepatocytes with 3T3-J2 fibroblasts. A "liver-on-a-chip" platform including HepG2/C3A cell spheroids was proposed for toxicological screening of substances and simulation of human liver pathologies [14]. At the same time, it is known that, unlike HepG2/C3A, HepaRG cell culture is more sensitive to drugs [16]. Moreover, polarized and specifically arranged differentiated HepaRG cells in spheroids can acquire a mature phenotype of hepatocytes and form intercellular contacts specific to human liver [15].

Cyclophosphamide (CP), one of the most widely used cytostatics, is relatively inactive, but exhibits a cytotoxic effect after biotransformation in the liver, *i.e.* is a prodrug [4]. It has been shown that *in vitro* hepatotoxicity of CP for 3T3-L1 mouse embryonic fibroblasts in the co-culture with primary human hepatocytes is higher than in the absence of liver cells [13].

We have previously shown that culturing of spheroids formed by HepaRG cells under dynamic

[1]Moscow Polytechnic University, Moscow, Russia; [2]M. P. Chumakov Federal Scientific Center for Research and Development of Immune-and-Biological Products, Russian Academy of Sciences, Moscow, Russia; [3]SRC BioClinicum Ltd., Moscow, Russia; [4]National Research University Higher School of Economics (HSE University), Moscow, Russia. ***Address for correspondence:*** pulkovanatalya@gmail.com. N. V. Pulkova

conditions of a microfluidic chip promotes expression of the main enzymes of xenobiotic metabolism [20], which is critical under conditions of CP treatment, because P450 cytochrome isoenzymes are responsible for its biotransformation and activation. Unlike liver cells, HaCaT cells do not metabolize CP.

In this work, we studied the cytotoxic effect of CP prodrug on epidermal cells (human HaCaT keratinocytes) and spheroids of human hepatoma HepaRG cells co-cultured in a microfluidic chip (BioClinicum). CP cytotoxicity was determined by absorption of neutral red (this method is used in the protocols of the Interdepartmental Coordination Committee for the validation of alternative methods) [9].

MATERIALS AND METHODS

3D culture of HepaRG cells was obtained by the hanging drop method in a medium containing William's E medium c GlutaMAX (Gibco), 10% fetal bovine serum (HyClone), 5 μg/ml recombinant human insulin (Gibco), 10^{-7} M sodium hydrocortisone hemisuccinate (HC, Sigma), and 1% penicillin/streptomycin (Gibco) [1]. The spheroids consisted of 5000 differentiated HepaRG cells and had a diameter of 200 μm.

To achieve a confluent monolayer, immortalized human HaCaT keratinocytes were cultured in DMEM with GlutaMAX (Gibco), 10% fetal bovine serum (HyClone), and 1% penicillin/streptomycin (Gibco).

Human keratinocytes and liver cells were co-cultured in a microfluidic chip equipped with a closed circuit of the nutrient medium circulation with two sequentially located cell wells [19]. To obtain a 2D culture, 7500 HaCaT cells were added to one well of a microfluidic chip and cultured for 48 h in a serum-free William's E medium with GlutaMAX (Gibco), 5 μg/ml of recombinant human insulin (Gibco), 10^{-7} M HC (Sigma), 5 ng/ml recombinant human epidermal growth factor (Gibco), 30 μg/ml bovine pituitary gland extract (Gibco), and 1% penicillin streptomycin (Gibco). Then, 20 spheroids of differentiated HepaRG cells were added to the second well of a microfluidic chip of the same contour. They were cultured for 48 h in a serum-free medium with or without CP (Sigma) at a micropump frequency of 1 Hz and a pressure of ±10 kPa. Under the same conditions, keratinocytes were cultured in the absence of liver cells with or without CP.

Cell viability was assessed by uptake of neutral red (NR, Sigma) according to the previously described method [9] or using the MTT assay (Sigma) [2]. Half-inhibitory concentration (IC_{50}) was determined by the Hill equation using the GraphPad Prism software (GraphPad Software). The viability of the HaCaT is presented as the ratio of optical densities in the presence and absence of CP.

All experiments were repeated in at least 3 independent replicates. The obtained results were processed using the Statistica 6.0 software (StatSoft, Inc.) and presented as $M \pm SD$. The data were compared using one-way ANOVA; the homogeneity of variances was checked using the Fisher's test. Significance of differences was assessed using the Student's t test at $p<0.05$.

RESULTS

It is known that about 20% CP in the systemic circulation is bound to blood plasma proteins (for some CP metabolites this binding is 60%) [10] Obviously, in order to avoid such nonspecific binding in the model system, it is advisable to carry out the cytotoxic tests with the culture medium in the absence of blood serum proteins. Moreover, the composition of the blood serum is not standardized, it is a complex mixture containing many components (cytokines, proteins, and inorganic salts) [18] which can complicate reproduction and interpretation of the results obtained. Therefore, toxicological studies of drugs are now more often carried out in serum-free media. For co-culturing of HepaRG and HaCaT cells, a serum-free medium of universal composition maintaining viability of these two cell lines is required. The most common approaches to optimization of the culture medium are based on both sequential changes in one or more of its components and the effect assessment on the growth or functional activity of cells [17]. We analyzed the compositions of the initial media maintaining proliferation of HepaRG and HaCaT cells to confluent monolayers and assessed the effect of changes in HC concentration on keratinocyte viability.

The media for culturing HepaRG and HaCaT cells to confluence under standard conditions are prepared on the basis of William's E and DMEM, respectively. According to the manufacturer's data they are similar in amino acid and salt composition. However, the

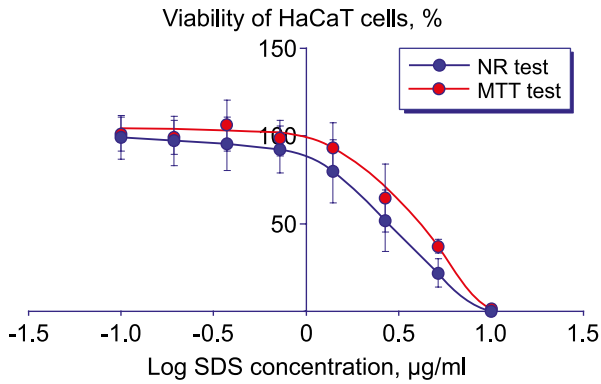

Fig. 1. Viability of HaCaT cells after 48-h incubation with SDS according to the results of NR test and MTT test.

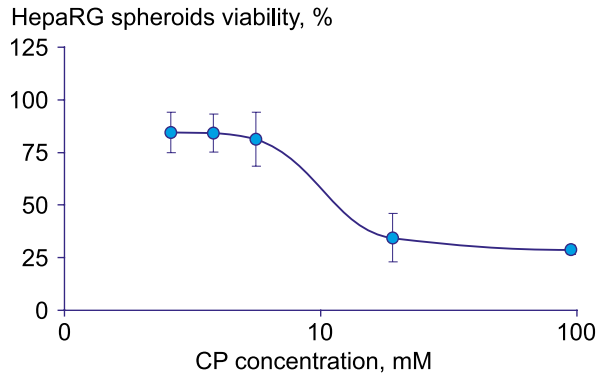

Fig. 2. The effect of CP on viability of HepaRG cells in 3D culture after 48-h incubation (NR test).

HepaRG cell culture medium additionally contains components that promote epithelial cell differentiation, which is also essential for keratinocytes (retinol and ergocalciferol), and other antioxidants such as tocopherol, ascorbic acid, and reduced glutathione. In addition, William's E medium contains menadione, a precursor of vitamin K, which can act as a prooxidant, as well as Ca^{2+} ions, as in DMEM medium, which is critical for keratinocyte differentiation [5]. Hence, it is more expedient to co-cultivate HepaRG and HaCaT cells in a more nutritious culture medium based on William's E and supplement it with hormones and growth factors, which is a standard approach in the use of culture media without the addition of serum (Biopredic, Life Technologies, PromoCell). Thus, recombinant human insulin and EGF were added to the universal culture medium, which are often used to maintain the growth of keratinocytes [21] and hepatocytes [3,18], as well as bovine pituitary gland extract, a powerful mitogen with high concentration of fibroblast growth factors and pituitary hormones [8]. It is known that addition of corticosteroid HC in a concentration of 10^{-7} M is critical for the maintenance of the phenotypic stability of HepaRG cell line [6]. To maintain proliferation of HaCaT cell a lower concentration of corticosteroids ($\leqslant 10^{-8}$ M) is used [7] to prevent the inhibition of lipid synthesis [12]. In the cytotoxic test with NR, no differences in HaCaT cell viability during incubation with a control substance SDS in a serum-free medium in the presence of 10^{-7} M HC or 2×10^{-9} M concentration of HC for keratinocyte culture) were found ($F=1.26$, $p=0.29$). Thus, co-culturing of HepaRG and HaCaT cells was carried out in a serum-free medium based on William's E containing 10^{-7} M HC, which is essential for the culture of HepaRG cells, as well as in the presence of recombinant human insulin, EGF, and an extract of bovine pituitary gland.

To select the optimal method for cell viability assessment, HaCaT cells were cultured in a serum-free medium with SDS in concentrations of 0.10, 0.19, 0.37, 0.72, 1.39, 2.68, 5.20, and 10 μg/ml. Cytotoxicity was analyzed by standard methods (MTT and NR tests). The NR test is based on the ability of live cells to absorb weak cationic dye NR that easily diffuses through the plasma and lysosomal membranes and binds electrostatically to anions of the lysosomal matrix. A toxi-

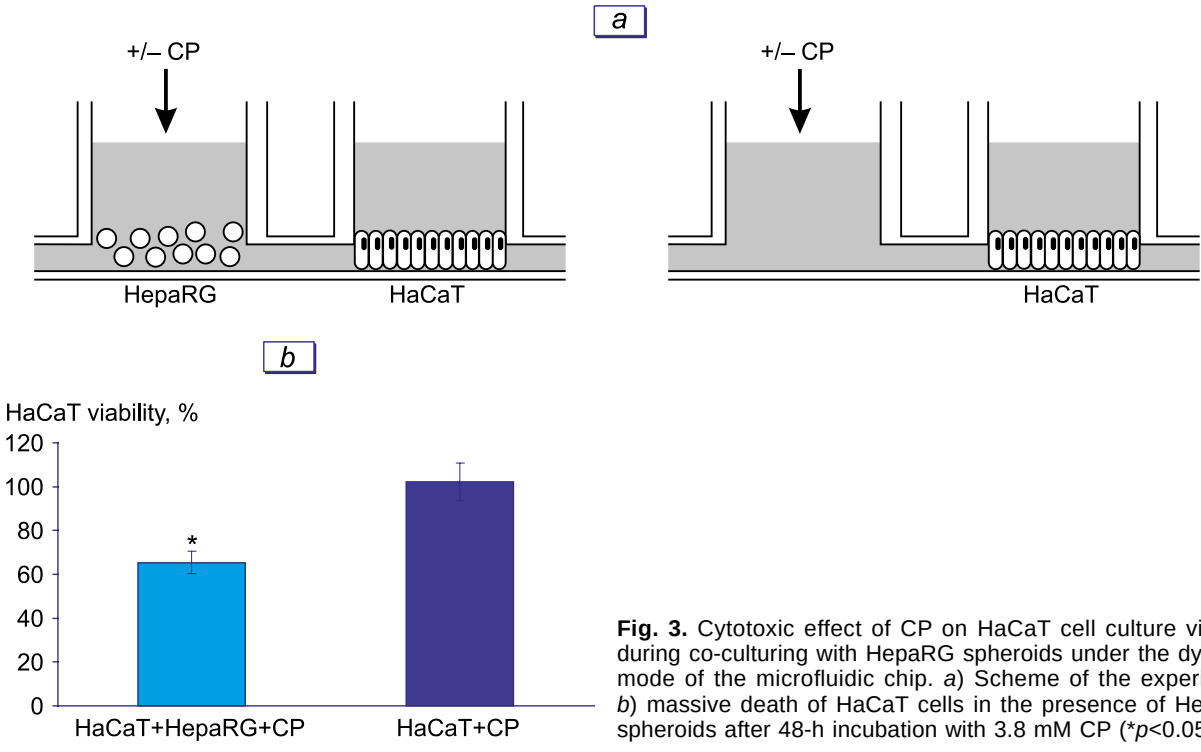

Fig. 3. Cytotoxic effect of CP on HaCaT cell culture viability during co-culturing with HepaRG spheroids under the dynamic mode of the microfluidic chip. *a*) Scheme of the experiment; *b*) massive death of HaCaT cells in the presence of HepaRG spheroids after 48-h incubation with 3.8 mM CP (*$p<0.05$).

cant, irrespective of the mechanism of action, reduces the rate of cell growth and the number of cells in culture. Cytotoxicity is expressed as a direct dependence of the degree of NR uptake by the cell culture on the concentration of the toxic substance. Another colorimetric method, the MTT assay, is based on the conversion of soluble MTT reagent, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide, into insoluble formazan catalyzed by mitochondrial SDH of live cells. MTT assay showed that SDS concentration inducing a 50% decrease in viability of the HaCaT cell culture (IC_{50}) after 48-h incubation is 4.8±1.2 μg/ml; while according to the NR test, IC_{50} for SDS was 3.2±1.1 μg/ml (Fig. 1), which correlates with previous data obtained for normal human keratinocytes [9]. The IC_{50} can be used in the regression formula to determine the doses causing mortality in laboratory animals in order to further classify substances according to the degree of danger to human health. The curves for the data set in the NR and MTT assays are different, F=7.91, $p<0.0001$. The NR test turned out to be more sensitive than the MTT assay. It is known that in the MTT assay, the formation of formazan crystals depends on the metabolic rate and the number of mitochondria in cells, which leads to greater errors in comparison with the NR test [22].

The NR test was used to assess hepatotoxicity of CP in concentrations of 2.6, 3.8, 5.6, 19, and 95 mM (Fig. 2). The IC_{50} of CP for HepaRG cell spheroids in culture after 48-h incubation in a serum-free medium was 13.1±1.4 mM. It was found that the concentration of 3.8 mM of CP reduced hepatocyte viability by less than 20% in comparison with culturing without CP (100±6%, $p<0.05$).

The use of a microfluidic chip made it possible to co-cultivate a 2D culture of HaCaT cells with spheroids of differentiated HepaRG cells under conditions of a circulating serum-free medium. The operating mode of the micropumps of the microfluidic chip corresponded to ±10 kPa; the impact of this pressure on HepaRG spheroids did not exceed physiologically permissible values, moreover, a high level of markers expression of the functional activity of liver cells similar to those *in vivo* was maintained under these conditions [20]. After 48-h incubation with 3.8 mM CP (the concentration did not much impair hepatocyte survival), the viability of HaCaT cells in co-culture with HepaRG spheroids decreased by about 40% in comparison with HaCaT cell monoculture exposed to CP (102±9%, Fig. 3). In the liver, CP is converted into 4-hydroxycyclophosphamide and aldophosphamide. These substances are non-toxic to cells and unstable, they are quickly converted into mustard phosphoramide and acrolein. The main cytotoxic metabolite in the cell is mustard phosphoramide. Acrolein is hepatotoxic, it enhances the cytotoxic effect of CP by forming conjugates with glutathione and depleting its pool in cells [4]. Obviously, the decrease in keratinocyte viability after co-culturing with hepatocytes in the presence of CP is due to the effect of metabolites formed in HepaRG spheroids as a result of CP biotransformation, released from liver cells, and exerting the toxic effect on cells distant from the site of their generation [13]. Interestingly, CP in a concentration of 3.8 mM produced no significant hepatotoxic effect during co-culturing of HepaRG cells with HaCaT a 3D culture under the dynamic conditions of a microfluidic chip, which can be explained by the influence of the nutrient medium circulation. Thus, the use of a microfluidic chip makes it possible to co-cultivate several types of cells, which is especially important for the analysis of cytotoxicity of prodrugs, *e.g.* CP, because culturing with hepatocytes provide conditions for drug activation and allows studying the cytotoxic effect of the drug and its metabolites on both liver cells and target organ cells, similar to *in vivo* conditions.

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