Toxicology in Vitro 29 (2015) 1897–1905

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

A novel micronucleus *in vitro* assay utilizing human hematopoietic stem cells

N. Kotova [a,*], N. Hebert [b,c,d], E.-L. Härnwall [a], D. Vare [a], C. Mazurier [b,c,d], L. Douay [b,c,d,e], D. Jenssen [a], J. Grawé [f,1]

[a] Stockholm University, Department of Molecular Biosciences, The Wenner-Gren Institute, SE-106 91 Stockholm, Sweden
[b] UPMC Univ Paris 06, UMR_S938 CDR Saint-Antoine, Prolifération et Différentiation des Cellules Souches, Paris, France
[c] INSERM, UMR_S938, Prolifération et Différentiation des Cellules Souches, Paris, France
[d] Etablissement Francais du Sang Ile de France, Ivry-sur-Seine, France
[e] AP-HP, Hôpital Saint-Antoine, Service d'Hématologie Biologique, Paris, France
[f] Dept. of Genetics and Pathology, Uppsala University, SE-751 85 Uppsala, Sweden

ARTICLE INFO

Article history:
Received 31 March 2015
Revised 10 June 2015
Accepted 20 July 2015
Available online 21 July 2015

Keywords:
Stem cells
Erythrocytes
Micronucleus test
Flow cytometry
Genotoxicity *in vitro*

ABSTRACT

The induction of micronucleated reticulocytes in the bone marrow is a sensitive indicator of chromosomal damage. Therefore, the micronucleus assay in rodents is widely used in genotoxicity and carcinogenicity testing. A test system based on cultured human primary cells could potentially provide better prediction compared to animal tests, increasing patient safety while also implementing the 3Rs principle, i.e. replace, reduce and refine. Hereby, we describe the development of an *in vitro* micronucleus assay based on animal-free *ex vivo* culture of human red blood cells from hematopoietic stem cells. To validate the method, five clastogens with direct action, three clastogens requiring metabolic activation, four aneugenic and three non-genotoxic compounds have been tested. Also, different metabolic systems have been applied. Flow cytometry was used for detection and enumeration of micronuclei. Altogether, the results were in agreement with the published data and indicated that a sensitive and cost effective *in vitro* assay to assess genotoxicity with a potential to high-throughput screening has been developed.

1. Introduction

Since 1959, the 3Rs rule, i.e. reduce, refine and replace, is the foundation of the ethical policy applied to animal testing in Europe and North America. Due to the vast number of chemicals and/or their mixtures to be assessed for safety, there is an increasing need to evaluate toxic effects at the cellular level as described e.g. in the term "21st century toxicology" (Berg et al., 2011). One of the challenges is to design appropriate tests which rely on a molecular and cellular mechanistic understanding of toxicants mode of action using *in vitro* methods based on human cells. Such an approach allows the evaluation of responses over a wide range of doses, providing an improved mechanistical basis for assessing human health risks associated with exposure to genotoxic and/or carcinogenic compounds. Moreover, it is well known that bioassays detecting genotoxicity in animals in some instances have limited biological relevance for humans as, for example, regarding different response to toxins in comparison to humans (Krewski et al., 2011). *In vivo* assays can also be laborious and time-consuming, as well as painful to animals.

For genotoxicity assessment, the micronucleus (MN) test in reticulocytes of bone marrow in rodents is one of the most applied tests *in vivo* (http://www.oecd-ilibrary.org). MN originate from whole chromosomes or parts of chromosomes having been separated from the main nucleus, and therefore reflect either chromosome breakage or impairment of the mitotic spindle. Mature red blood cells lack DNA and RNA while reticulocytes still contain RNA. Therefore, these cells with induced MN containing DNA fragment(s) can easily be identified by flow cytometry (FCM) (Heddle et al., 2011).

Previously, we have developed a highly sensitive method to determine the frequency of micronucleated reticulocytes *in vivo* in rodents based on dual-laser FCM (Grawe et al., 1992). Using this

Abbreviations: MN, micronuclei; FCM, flow cytometry; RBCs, red blood cells; LK, leukapheresis; CB, cord blood; IMDM, Iscove Modified Dulbecco's Medium; SCF, stem cell factor; IL-3, interleukin-3; Epo, erythropoietin; DBPD, dibenzo[a,l]pyrene-11,12-dihydrodiol; DBPDE, dibenzo[a,l]pyrene-11,12-dihydrodiol-13,14-epoxide; DMBA, 7,12-dimethylbenz(a)antracene.

* Corresponding author.
E-mail address: Natalia.Kotova@su.se (N. Kotova).
[1] Present address: Q-linea AB, Dag Hammarskjölds väg 54B, SE-75237 Uppsala, Sweden.

http://dx.doi.org/10.1016/j.tiv.2015.07.018
0887-2333/

methodology, we achieved an improvement in sensitivity by a factor of about 10, as compared to manual microscopic scoring (Abramsson-Zetterberg et al., 1995; Grawe et al., 1998, 1992). The possibility to automate the evaluation of *in vivo* MN-test by FCM has further increased the application of this method in genotoxicity testing (Hayashi et al., 2000, 2007). Detailed method protocols have therefore been published (Dertinger et al., 2003; Grawe, 2005). For assessing cytogenetic damage, FCM-scoring of micronucleated erythrocytes in rodents is now a well-established and efficient platform which is highly predictive towards carcinogenicity, relatively easy to evaluate and contains improved technical advantages (Dertinger et al., 2011). The strength of this test is that it exclusively detects MN arising in the bone marrow and thus indicates that the genotoxic substance tested is effective in that tissue. Because the hematopoietic cells undergo rapid division, the test is highly sensitive to genotoxic agents as well as to aneugenic agents that produce changes in the chromosome number. The MN-test in newly-formed erythrocytes has also been applied by us in studies on humans (Abramsson-Zetterberg et al., 2000; Kotova et al., 2014; Stopper et al., 2005) as well as by others (Flanagan et al., 2010; Offer et al., 2005).

Based on our previous knowledge and experience in technical development of the MN-test, we have developed a non-animal genotoxic test *in vitro* which is analogous to micronucleus test *in vivo*. For this purpose, we have exploited the *ex vivo* generation of human erythrocytes from hematopoietic stem cells in conditions with no animal components as previously described (Giarratana et al., 2011). First, we showed that it is possible to induce and monitor MN induction by genotoxic agents in *ex vivo* generated human reticulocytes. Thereafter, in order to validate the method, a panel of clastogenic agents with direct action (γ-radiation, mitomycin C, methyl methansulfonate, etoposide, dibenzo(a,l)pyrene-11,12-di hydrodiol-13,14-epoxideand), clastogens requiring metabolic activation (7,12-dimethylbenz(a)antracene, benzo(a)pyrene, cyclophosphamide monohydrate, dibenzo(a,l)pyrene-11,12-dihy drodiol) as well as aneugens (colcemid, griseofulvin, vincristine sulfate, bisphenol A) and non-genotoxic compounds (benzyl alcohol, ethylenediaminetetraacetic acid, sodium chloride) has been tested. For metabolic activation, rat S9 mix and human S9 mix as well as co-culture with HepaRG and XEM2 cell lines have been applied.

2. Materials and methods

2.1. Cells and culture conditions

2.1.1. Cultured red blood cells (RBCs)

The culture conditions described here allow highly selective differentiation of CD34+ hematopoietic stem cells into fully matured RBCs. CD34+ cells have been isolated either from cord blood (CB) cells or leukapheresis (LK) from healthy individuals once informed consent obtained.

CD34+ cells were isolated by supermagnetic microbead selection by the use of Mini-MACS columns (Miltenyi Biotec; 94% ± 3% purity). The cells were cultured in the presence of Iscove Modified Dulbecco's Medium (IMDM) supplemented with stabilized glutamine (Biochrom) containing 330 µg/mL human holo-transferrin (Scipac), 10 µg/mL recombinant human insulin (Incelligent SG; CellGen), 2 IU/mL heparin (Choay), 5% solvent/detergent virus-inactivated human plasma (Etablissement Français du Sang) and with sequential addition of 100 ng/mL stem cell factor (SCF; PeproTech), 5 ng/mL interleukin-3 (IL-3; PeproTech) and 3 IU/mL erythropoietin (Epo; Eprex). On day 4, one volume of cell culture was diluted in four volumes of fresh medium containing SCF, IL-3 and Epo. On day 8, the cells were resuspended at 10^5 cells/mL in fresh medium supplemented with SCF and Epo. In the second phase (day 11–day 18), the cells were adjusted to 1×10^6 cells/mL and cultured in fresh medium supplemented with Epo alone. By day 15, the mean cell amplification of CD34+ cells reached a plateau of approximately 30000-fold for LK and 100000-fold for CB cells.

2.1.2. Metabolic activation

For metabolic activation, one part of rat S9 liver postmitochondrial fraction (S9) (MOLTOX, Trinova Biochem, Germany) and 1.5 parts cofactor mixture were used. The cofactor mixture was ß-nicotinamid adenine dinucleotide phosphate sodium salt and glucose-6-phosphate sodium salt dissolved in IMDM (final concentration in cell culture was 0.6 mmol/L and 3.75 mmol/L respectively). As an alternative, human S9 (XenoTech, USA) with the same cofactor mixture as for rat S9 was applied. XEM2 cells were grown in Dulbeccos modified Eagle's media (DMEM) containing 9% serum and 0.9% penicillin/streptomycin, in 37 °C at 5% CO_2. The medium for HepaRG cells (Biopredic International, France) was prepared according to user guide from a supplier. For co-culture with HepaRG or XEM2 cells in inserts, HTS Transwell-24 well plates with 0.4 µm polycarbonate membrane were used (Corning Incorporated, USA).

2.2. Compounds and γ-radiation source

The compounds used were paraformaldehyde (Sigma–Aldrich, Germany, CAS no 30525-89-4), May-Grünwald solution (Merck, Germany), Giemsa solution (Merck, Germany), phosphate-buffered saline (PBS; Sigma–Aldrich, Germany), glucose-6-phosphate sodium salt (Sigma–Aldrich, Germany, CAS no 54010-71-8), ß-nicotinamid adenine dinucleotide phosphate sodium salt (Sigma–Aldrich. Germany, CAS no 698999-85-8), GlutaMax (LifeTechnologies, USA), Iscove's Modified Dulbecco's Medium (IMDM, Sigma–Aldrich, Germany), dimethylsulfoxide (DMSO, Scharlau, CAS no 67-68-5), Hoechst 33342 (Sigma–Aldrich, Germany, CAS no 23491-52-3), Thiazole orange (Sigma–Aldrich, Germany, CAS no 107091-89-4). Compounds tested are listed in Table 1. The final concentration of DMSO or water in complete medium did not exceed 0.1% (v/v). In all cases of γ-radiation, the cells were irradiated with a single acute dose by ^{137}Cs source at the dose-rate of 0.37 Gy/min.

2.3. Treatment schedule

2.3.1. Compounds with direct action

During the initial experiments, the cells were treated on days 11–15 in order to test different culture conditions. The cells were transferred from T-flasks into 24-wells plates in concentration 1×10^6 cells per mL per well in a final volume of 1 mL per well. For testing in 96-wells plates, a final volume was 0.2 mL per well. To test a short-term or prolonged treatment to genotoxic agents with a direct mechanism of action, i.e. without metabolic activation, cells were treated for 1 h or 24 h and had a recovery time of 24-, 48-, 72- or 120 h. After the treatment, the cells were washed twice, first time with 9 ml and then with 7 ml PBS.

2.3.2. Compounds that require metabolic activation

For testing of genotoxic agents which require metabolic activation, cells were treated with a respective chemical for 3 h (short-term exposure) or 24 h (prolonged treatment) with final concentration of 1% or 2% microsomal liver cell fraction from rat (S9 mix) followed by 69- or 48-h recovery time, respectively. For tests with human S9 mix, cells were treated with a respective chemical for 3 h with final concentration of 1%, 2%, 5% or 10% mix followed by 69-h recovery time. XEM2 and HepaRG cells were