The MTT assay is not adequate for the assessment of mutated PAI-1 toxicity towards endothelial cells

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Abstract

Present study was aimed at the evaluation of the endothelial cytotoxicity of the plasminogen activator inhibitor type-1 mutated protein characterized by the very long half-life time (VLHL PAI-1) and therefore prolonged anti-proteinase activity. Viability of the human umbilical vein endothelial cells (HUVECs) cultured for 72 hours with the increasing concentrations (1, 10, 100 µg/ml) of VLHL PAI-1 was not affected as demonstrated by the neutral red test and direct cell counting. On the contrary, the classical MTT test proved to be unreliable for this experimental setting most possibly due to the cell specific VLHL PAI-1 – HUVECs interaction. This suggestion was further confirmed by the similar results of the viability assessment by the MTT test and direct cell count in the A549 and NCI-H1299 lung cancer cell lines cultures with VLHL PAI-1 mutated protein.

***Key words:** PAI-1, cytotoxicity, MTT test, neutral red assay, HUVEC.*

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Introduction

Plasminogen activator inhibitor type-1 (PAI-1) the primary regulator of plasminogen system biological activity, is essential part of the sophisticated system coordinating cell adhesion and migration. It also plays important part in the angiogenesis processes, mostly by affecting endothelial cells and therefore sprouting and tube formation stages of new blood vessels formation [1]. Our previous studies have demonstrated that proteolytic activity plays important part in its anti-angiogenic effect as observed in the sprout formation assay [2]. To analyze further the PAI-1 effect on the endothelial cells we decided to assess its possible cytotoxicity. Here, we describe the different sensitivity and applicability of two different quantitative colorimetric assays – neutral red and MTT test classically used for the experimental evaluation of examined substances effect on cells viability. As opposed to the A549 and H1299 lung cancer cell lines, MTT test was proven unreliable for the assessment of PAI-1 effect on the endothelial cells represented by the human umbilical vein endothelial cells line.

Material and Methods

PAI-1 protein

VLHL PAI-1 molecule produced by the mutation of two amino acids (Gln197→Cys, Gly355→Cys) characterized by the very long half-life of over 700 h (VLHL) was constructed, expressed in E. coli and purified as we described in details before [2, 3]. Briefly, the cDNA encoding PAI-1 was excised from the VLHL PAI-1 plasmid as an *Nde*I/*XhoI* fragment. All mutations were introduced by PCR. The PCR product of the VLHL PAI-1s *Nde*I/*XhoI* fragment was ligated into the pFastbac plasmid, which contains a 6His purification tag. A bacmid containing VLHL PAI-1 DNA was used to transfect Sf9 cells deri-

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ved from *Spodoptera Frugiperda* (Fall Armyworm, USA) using cell-lectin reagent (Invitrogen,USA), according to the manufacturer's instructions. The virus produced by the cells was then used to infect Sf9 cells. The flasks were incubated in a rotary incubator for 72 hrs at 27˚C, harvested, and lysed by two freeze-thaw cycles. The lysate was centrifuged at 3 000 × g for 20 minutes to pellet cellular debris. The supernatant was loaded onto a nickel resin-packed column (Invitrogen, Carlsbad, USA). The peak fractions were further purified on HPLC Superose 12 FPLC column (Millipore, Germany).

Cell culture

The human umbilical vein endothelial cells (HUVEC) line was purchased from Cambrex Inc., (East Rutherford, NJ, USA). Cells were grown to confluency in the EGM-2MV media (Cambrex Inc, USA) in a humidified atmosphere at 37˚C, 5%CO_2 (Sony, Japan). Cells were trypsinized in solution of 0.05% trypsin and seeded into 96-well microplates at the density of 10 000 cells/well.

The human lung cancer cell lines A549 (CCL-185) and NCI-H1299 (CRL-5803) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown to confluency: A549 in the Ham's F12 medium with 2mM L-glutamine adjusted; NCI-H1299 in the RPMI medium with 2mM L-glutamine adjusted plus 10% fetal bovine serum and Antibiotic-Antimycotic (Gibco, Germany) in a humidified atmosphere at 37˚C, 5% CO_2. Cells were trypsinized in solution of 0.05% trypsin and seeded into 96-well microplates at the density of 5000 cells/well.

Afterwards, cells were grown for 24 hours to the subconfluent state, subsequently fresh medium containing the different concentrations of VLHLPAI-1 1, 10, 100 µg/ml was added to triplicate wells. Treated cells were incubated for 72 hours at 37˚C, 5%CO_2.

Cell viability

Automatic cell counting

Cells were trypsinized, suspended in the PBS and counted using the Coulter automatic cell counter. Fraction of viable cells in cultures with VLHL PAI-1 was calculated as a percentage of viable cells in control cultures.

MTT assay

The MTT assay was based on the method of Mossman performed according to Vian modification [4, 5]. Briefly, to each well was added 50 µl medium containing 10 mg MTT/ml (Sigma-Aldrich, Poland), and the microplate was incubated at 37˚C, 5% CO_2 for 4 hours. At the end of incubation period 0.1 ml DMSO (Sigma-Aldrich, Poland) was added to each well. The plate was shaken on the microplate shaker to dissolve the blue MTT-formazan. Absorbances were read at the 492 nm on a microplate reader. Results obtained in the consecutive cultures with VLHL PAI-1 were presented as a percentage of absorbance in control cultures.

Neutral red assay

The neutral red (NR) assay based on the method of Borenfreund and Purner was performed using the In Vitro Toxicology Assay Kit Neutral Red Based (Sigma-Aldrich, Poland) [6]. Briefly, neutral red solution (500 µg/ml) was added to each well in an amount equal to 10% of the culture medium volume (10 µl to 100 µl) and the microplate was incubated at 37˚C, 5% CO_2 for 2 hours. At the end of incubation period, the medium was removed and cells were rinsed with fixative. Afterwards, 100 µl of solubilization solution was added to each well equal to the original volume of culture medium. Absorbances were read at the 540 nm on a microplate reader. Results for consecutive cultures with VLHL PAI-1 were presented as a percentage of absorbance in control cultures.

Results

Viability of the endothelial cells (HUVECs) cultured for 72 hours with increasing concentrations of VLHL PAI-1 cells as assessed by the classical MTT assay was significantly different to the outcomes shown by the neutral red assay (Tab. 1).

MTT in comparison to neutral red test demonstrated significantly lower viability of endothelial cells cultured with VLHL PAI-1 in concentrations of 1 µg/ml ($p<0.01$) and 10 µg/ml ($p<0.05$), and increased number of cells cultured with 100 µg/ml ($p<0.01$) of the mutated protein.

Table 1. Viability assessed for different cell lines by means of classical MTT assay and neutral red assay (HUVEC cells only) as compared to the cell counting method. Statistical difference was calculated by t-Student test for the results of HUVECs viability

Cell line	VLHL PAI-1 (µg/ml)	Cells viability (%)		
		Neutral red assay x±SD	MTT assay x±SD	Cell counting
NCI-H1299	1	-	103.00±2.83	102
	10	-	92.85±8.27	105
	100	-	67.00±14.14	64.7
A549	1	-	102.50±2.12	95.8
	10	-	90.10±10.04	94.7
	100	-	80.70±10.89	79.4
HUVEC	1	92.74±9.48	71.5±6**	101
	10	92.30±11.35	69.5±14*	97
	100	80.05±10.19	123.5±13**	80.5

** p<0.05, ** p<0.01*

The neutral red test did not show any significant effects of VLHL PAI-1 on the endothelial cells viability. Similarly, automatic cell counting of endothelial cells from the parallel cultures did not prove any interference of VLHLPAI-1 with cells viability.

On the contrary to the HUVECs, MTT assessed viability in two lung cancer cell lines, NCI-H1299 human non-small cell lung cancer and A549 human lung epithelial adenocarcinoma, cultured for 72 hours with increasing concentrations of VLHLPAI-1, proved to be comparable to the results of automatic cell counting performed in the parallel cultures.

Discussion

MTT colorimetric cell viability assay is considered the classical test allowing the quantification of the potential cytotoxicity of examined compound. Cells are exposed to the various concentration of researched agent and their survival is assessed on the basis of the mitochondrial enzymes ability to chemically reduce the soluble yellow tetrazolium salt (MTT) to an insoluble formazan dye. The MTT assay, initially developed by Mossman has undergone many modifications because of its less than optimal sensitivity and the difficulty in solubilizing the final formazan product [4]. It is however extensively used as a convenient and rapid measure of cell viability, also due to its simplicity and cost-effectiveness.

There is a considerable number of studies utilizing the MTT test for the assessment of HUVEC cells viability, proliferation or cytotoxic effect of certain compounds [7, 8]. Therefore, the failure of the MTT test to reliably reflect the HUVEC cells viability that we are reporting in present study could not be related to the cell-type specific inefficiency of endothelium to metabolize tetrazolium salt as it was reported for example for L929 fibroblasts [5]. In our opinion, it might rather reflect direct or indirect VLHL PAI-1 impact on the endothelial cells function. The utterly contradictory effects of increasing PAI-1 doses on HUVEC viability – negative for 1 μg/ml (71.5±6% of control) and 10 μg/ml (69.5±14% of control) while positive for high concentration of 100 μg/ml (123.5±13% of control) further confirms the failure of endothelial cells to effectively metabolize MTT and undergo the test. Therefore, cited results should be definitely regarded as an artifacts. HUVEC-related specificity of observed MTT discrepancies has been further confirmed by experiments involving VLHL PAI-1 and cancer cell lines. In both settings, matching results of the MTT test and direct cell count were observed. As mentioned above, mitochondrial enzyme succinic dehydrogenase is responsible for the tertrazolium salt reduction into the formazan product. Thus, the most obvious explanation for the observed phenomenon might be either direct or indirect interaction between PAI-1 and mitochondria metabolic status involving changes in succinic dehydrogenase activity. Also, our results point toward intracellular effects of the HUVECs exposure to VLHL PAI-1, as the results of the neutral red test proved both structural and functional stability of endothelial cell membrane. In opposition to the MTT test, the neutral red viability assay is based on the incorporation of the supravital dye (neutral red) into the lysosomes of viable cells after their incubation with examined substances. Weakly cationic dye penetrates cell membranes by the nonionic diffusion and binds intracellulary to sites of the lysosomal matrix [9]. Dead or damaged cells cannot retain the dye after the washing and fixation procedures. Therefore, the process of dye acquisition is not in any way due to the cell metabolic activity, as it is in the MTT test. We have also demonstrated that results of cells viability assessed by the neutral red test were in agreement with direct count outcomes. This observation further confirms our suggestion of VLHL PAI-1 – HUVECs interaction affecting cell metabolic status and therefore responsible for the inapplicability of the MTT test in this particular experimental setting.

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References

1. Chorostowska-Wynimko J, Skrzypczak-Jankun E, Jankun J (2004): Plasminogen activator inhibitor type – 1 – structure and biological activity. Int J Mol Med 13: 759-766.
2. Chorostowska-Wynimko J, Swiercz R, Skrzypczak-Jankun E et al. (2003): A novel form of the plasminogen activator inhibitor created by cysteine mutations extends its half-life: relevance to cancer and angiogenesis. Mol Cancer Ther 2: 19-28.
3. Jankun J, Aleem AM, Selman SH et al. (2007): Highly stable plasminogen activator inhibitor type one (VLHL PAI-1) protects fibrin clots from tissue plasminogen activator-mediated fibrinolysis. Int J Mol Med 20: 683-687.
4. Mosmann T (1983): Rapid colorimetric assay for cellular growth and survivals: application to proliferation and cytotoxicity assays. J Immunol Meth 65: 55-63.
5. Vian L, Vincent J, Maurin J et al. (1995): Comparison of three in vitro cytotoxicity assays for estimating surfactant ocular irritation. Toxicol in Vitro 9: 185-190.
6. Borenfreund E, Puerner JA (1985): Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicol Lett 24: 119-124.
7. Pyriochou A, Tsigkos S, Vassilakopoulos T et al. (2007): Anti-angiogenic properties of a sulindac analogue. Br J Pharmacol 152: 1207-1214.
8. Cianchetti S, Del Fiorentino A, Colognato R et al. (2008): Anti-inflammatory and anti-oxidant properties of telmisartan in cultured human umbilical vein endothelial cells. Atherosclerosis 198: 22-28.
9. Babich H, Borenfreund E (1987): Structure-activity relationship (SAR) model established in vitro with the neutral red cytotoxicity assay. Toxicol in Vitro 1: 3-9.