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Brief Report

Evaluation of lymphocytes inactivation by extracorporeal photopheresis using tetrazolium salt based-assay

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ABSTRACT

Extracorporeal photopheresis (ECP) is accepted as a second-line therapy for the treatment of acute and chronic steroid-refractory graft versus host disease (GvHD), cutaneous T-cell lymphoma and solid organ transplantation.

ECP should be validated: we compared in parallel apoptosis and proliferation analysis of patient lymphocytes treated with 8-MOP ECP using respectively Annexin V/7-aminoactinomycin D (7-AAD) and CFSE with a tetrazolium salt (WST-1) method. Using WST-1 assay we found a significant decrement ($p < 0.01$) of metabolic activity at 4 days between ECP-treated and untreated cells. This finding was confirmed by the significant decrease of cell proliferation and increase of cell death observed by CFSE and 7AAD-Annexin V, respectively. Accordingly, once validated against a reference method, WST-1 could represent a rapid and easy assay for routinely quality control of ECP.

1. Introduction

Extracorporeal photopheresis (ECP) is accepted as a second-line therapy for the treatment of acute and chronic steroid-refractory graft versus host disease (GvHD) [1], cutaneous T-cell lymphoma [2] and solid organ transplantation [3].

The clinical effect is based on the induction of apoptosis or proliferation inhibition by ultraviolet A light (UVA)-activated 8-methoxypsoralen (8-MOP) on patient lymphocytes.

Currently, on-line and off-line systems are available. In the former, irradiation and reinfusion are performed in one step. In the latter, MNC are irradiated separately and then reinfused.

Validation of ECP protocol is requested for clinical use giving evidence of the biological effect on cell proliferation arrest and death.

However, the best validation protocol has not yet been established and few studies have addressed the problem. In 2003, Jacob et al. [4] evaluated ECP by quantifying the inhibition of mitogen-induced proliferation by using radioactive 3-H thymidine. They observed an inhibition over 90% in as many as 94% of the cases. Subsequently, Evrard et al. [5] evaluated the inhibition of mitogen-induced proliferation after ECP both using 3-H thymidine and a non-radioactive CarboxFluorescein Succinimidyl Ester (CFSE) in flow cytometry. They concluded that the two methods were comparable. More recently, Faivre et al. [6] validated the ECP by using a proliferation assay based on CFSE in flow cytometry according to ISO 15189:2007 Standard. A good concordance between the CFSE and the 3-H thymidine assays was reported.

In 2014, Taverna et al. [7] performed the ECP quality control in flow cytometry by evaluating the induction of

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lymphocyte apoptosis generated after ECP on 13 patients with chronic GvHD by using annexin V/propidium iodide staining. Inhibition of mitogen-induced proliferation by CFSE was also measured. With Annexin V/PI a faster and easier measurement was made possible compared to CFSE. Overall, it seems that quantification of cell proliferation by CFSE and the evaluation of cell apoptosis using annexin V/propidium iodide may be a valid alternative to methods based on the use of 3-H thymidine; however, both methods require the use of a flow cytometer restricting their use to centers with access to this technology.

In this study, we compared in parallel two established methods, namely apoptosis and proliferation analysis of patient lymphocytes treated with 8-MOP ECP using respectively Annexin V/7-aminoactinomycin D (7-AAD) and CFSE with a commercially available tetrazolium salt (WST-1) method. WST-1 permits to quantify metabolically active cells by correlating the number of these cells with the amount of formazan derived by the cleavage of WST-1 by mitochondrial dehydrogenases.

2. Materials and methods

Laboratory instrumentation was subjected to installation and operational qualification and analytical methods were validated. MNC were collected from six patients with chronic GvHD by using a COBE SPECTRA apheresis system and ECP was performed by using the off-line Vilbert–Lourmat method: cells were irradiated with UVA light for 10 minutes adjusted to 2 J/cm^2 (Macogenic, Macopharma, France) in the presence of 200 ng/ml of 8-MOP.

For WST1 test, before and after UVA irradiation, 100,000 cells were plated in quadruplicate in 96 well plate. Immediately after treatment and after 2 and 4 days of culture, 10 μl of WST-1 was added in each well; after 4 hours of incubation, the absorbance of the dye was measured by ELISA. Results were expressed as: 100 × (absorbance of sample post-ECP/absorbance pre-ECP).

For CFSE assay, 200,000 cells were resuspended in RPMI, 10% FBS, 1% L-glutamine, labeled for 10 minutes at 37 °C, 5% CO_2 with CFSE (5 μM) before and after irradiation and then stimulated with IL2 (500 U/ml) and anti-CD3 (500 ng/ml). After 4 days, an analysis of the CFSE staining on CD45+ cells and T-cell subpopulations (CD5+/CD4+; CD5+/CD8+; CD5–/CD56+) was performed by flow cytometry. The inhibition of cell proliferation induced by the treatment was calculated as follows: 100 × (%CFSE preECP – %CFSE postECP)/%CFSE preECP.

For 7AAD-Annexin V analysis, 200,000 cells were seeded in 96 well plate to quantify early and late apoptosis immediately after irradiation and after 2 and 4 days of cell culture. Cell subpopulations were analyzed as described above. Result was expressed as follows: Δapoptosis = percentage of 7AAD(+)ANN V(+) cells postECP – %7AAD(+)ANN V(+) preECP.

To evaluate if the differences between preECP and postECP treatment were statistically significant, the paired Student's t test was used. $P \leq 0.05$ was considered significant.

3. Results

The inhibition of the mitogen-induced proliferation 4 days postECP treatment measured by CFSE on CD45+ cells was 94.7 ± 2.5% (range: 90.6–97.7, n = 6; Fig. 1A). No differences were observed between the three cell subpopulations investigated (CD4+ lymphocytes, CD8 + T cells and NK cells) revealing that T and NK cells were equally affected by ECP treatment (data not shown).

Increment of cell apoptosis measured on CD45+ 7AAD+/Annexin V+ cells at day 4 postECP was 19.4 ± 14.1% with respect to untreated cells ($p < 0.05$) and total apoptotic cells postECP was 98.1 ± 2.4 (range: 94 .7–100.0, n=4) (Fig. 1B). Furthermore, no differences in cell apoptosis and death were observed between the three cell subpopulations investigated (data not shown). For WST-1, decrement of enzymatic activity in ECP samples 4 days after treatment was 65.5 ± 15.5% (range: 83.0–50.1, n = 4) with respect to untreated cells (Fig. 2a).

4. Discussion

Centers performing hematopoietic stem cell transplantation accredited under FACT/JACIE International standards (http://www.jacie.org/standards) are recommend to validate each manipulation performed in the processing facility. For ECP, a functional test showing the induction of cell proliferation arrest and death should be performed in vitro to reproduce the effects ascribed to 8-MOP activation. Validation criteria however have not been yet established. The Italian Society of Hemapheresis and Cell Manipulation and Italian Group for Bone Marrow Transplantation proposed that the percentage of CD3+/7-AAD+ lymphocytes after 72–96 hours of culture should be not less 50% [8], and Taverna et al. [7] proposed to set a Δapoptosis "alerting" threshold, defined as the difference between the % of annexin V positive cells in the pre and post ECP, of at least 15%.

The quantification of CFSE and 7AAD-Annexin V using a flow cytometry are preferable because they can measure parameters as proliferation, apoptosis, viability, immunophenotype, and morphology simultaneously whereas WST-1 assay permits the quantification of the metabolically intact cells. However, the cytofluorimetric method requires skilled operators and a flow cytometer is not easily available.

Using WST-1 assay we found a significant decrement ($p < 0.01$) of metabolic activity at 4 days between ECP-treated and untreated cells. This finding was confirmed by the significant decrease of cell proliferation and increase of cell death observed by CFSE and 7AAD-Annexin V, respectively. In addition, WST-1 assay does not require washing and harvesting of cells, and it is entirely performed in the same microplate used for the ELISA reader. Accordingly, once validated against a reference method, WST-1 could represent a rapid and easy assay for routinely quality control of ECP not requiring the use of a flow cytometer or radioactive compounds. A minimum threshold of Δapoptosis, evaluated with this approach, should be established by further studies.

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