NCL Method ITA-17

Analysis of Nanoparticle effects on Leukocyte Procoagulant Activity

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This protocol assumes an intermediate level of scientific competency with regard to techniques, instrumentation, and safety procedures. Rudimentary assay details have been omitted for the sake of brevity.

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1. Introduction

Leukocyte procoagulant activity (PCA) is accepted as an important component in the onset of disseminated intravascular coagulation (DIC). DIC is common in acute promyelocytic leukemia (APL) and other forms of cancer [1-5]. DIC in cancer patients is often observed after initiation of therapy with cytotoxic oncology drugs that act by altering DNA replication (e.g., doxorubicin, daunorubicin, and vincristin) [3, 6]. Cytotoxic oncology drugs acting by other mechanisms, (e.g., methotrexate and paclitaxel) do not induce DIC [7-8]. DIC is also a common complication in sepsis [9-12]. Cytotoxic drugs (doxorubicin, daunorubicin and vincristin) and endotoxin have previously been shown to induce leukocyte PCA *in vitro* and DIC *in vivo* [13-21]. *In vitro*, doxorubicin-induced leukocyte PCA has previously been linked to DIC *in vivo* [3].

2. Principles

This document describes a protocol for assessing the ability of a nanoparticle formulation to induce leukocyte procoagulant activity. This protocol includes two cell models: normal leukocytes, represented by PBMC isolated from blood of healthy donor volunteers, and acute promyelocytic leukemia cells, represented by the HL-60 cell line. Briefly, cells are treated with nanoparticles, and then undergo a wash step to remove excess particles. Isolated cells are then used to initiate plasma coagulation, which is measured using coagulometer (following NCL protocol ITA-12) for analysis of prothrombin time.

3. Reagents, Materials, Cell Lines, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; these reagents were used in the development of the protocol and their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted. Please note that suppliers may undergo a name change due to a variety of factors. Brands and part numbers typically remain consistent but may also change over time.

3.1 Reagents

3.1.1 Human blood from at least 3 donors, anti-coagulated with Li-heparin for PBMC isolation and anti-coagulated with Na-citrate for plasma coagulation test

3.1.2 Ficoll Paque Premium (GE Healthcare, 17-5442-02)

3.1.3 Phosphate buffered saline (PBS) (GE Life Sciences, Hyclone, SH30256.01)

3.1.4 Ultrapure LPS (InvivoGen, tlrl-peklps)

3.1.5 Doxorubicin Hydrochloride (Bedford Labs, NIH DVR pharmacy)

3.1.6 Fetal bovine serum (FBS) (GE Life Sciences, Hyclone , SH30070.03)

3.1.7 RPMI-1640 (GE Life Sciences, HyClone, SH30096.01)

3.1.8 Hank's balanced salt solution (HBSS) (Gibco, 14175-095)

3.1.9 Penicillin streptomycin solution (GE Life Sciences, Hyclone, SV30010)

3.1.10 L-glutamine (GE Life Sciences, Hyclone, SH30034.01)

3.1.11 Trypan Blue solution (Gibco, 15250-061)

3.1.12 Neoplastine Cl Plus (Diagnostica Stago, 00667)

3.1.13 CoagControl N+ABN (Diagnostica Stago, 00677)

3.1.14. Calcium ionophore (Sigma-Aldrich, C7522)

3.2 Materials

3.2.1 Pipettes covering the range of 0.05 to 10 mL

3.2.2 6-well plates

3.2.3 Polypropylene tubes, 50 and 15 mL

3.2.4. Finntip, 1.25 mL (ThermoScientific, NC0298434)

3.2.5. 4-well cuvettes (Diagnostica Stago, 38876)

3.2.6 Polystyrene tubes, 5mL (Falcon, 352058)

3.2.7 Scintillation vials, 20mL (Research Products International, FS74511-20)

3.3 Cell Lines

3.3.1 HL-60 promyelocytic cells (ATCC, CCL-240)

3.4 Equipment

3.4.1 Centrifuge

3.4.2 Refrigerator, 2-8ºC

3.4.3 Freezer, -20ºC

3.4.4 Cell culture incubator with 5% CO_2 and 95% humidity.

3.4.5 Biohazard safety cabinet approved for level II handling of biological material

3.4.6 Inverted microscope

3.4.7 Vortex

3.4.8 Hemocytometer

3.4.9 Coagulometer STart4 (Diagnostica Stago) or comparable

4. Reagent and Control Preparation

4.1 Complete RPMI-1640 medium

The complete RPMI medium should contain the following reagents:

FBS (heat inactivated) 10% for PBMC and 20% for HL-60

2 mM L-glutamine

100 U/mL penicillin

100 µg/mL streptomycin sulfate

Store at 2-8ºC protected from light for no longer than 1 month. Before use, warm in a water bath.

4.2 Lipopolysaccharide, 1 mg/mL (LPS, Stock)

Add 1 mL of sterile PBS or cell culture medium per 1 mg of LPS to the vial and vortex to mix. Store daily use aliquots at a nominal temperature of -20°C.

4.3 Doxorubicin, stock

Doxorubicin (adriamycin) is provided as a solution at 2 mg/mL and stored at 2-8ºC.

4.4 Positive Controls for cell culture

Positive control for PBMC

Dilute stock LPS solution in cell culture medium to a final concentration of 1 µg/mL. Store at room temperature. Discard unused portion after experiment. Alternatively, calcium ionophore at the final concentration of 50 ng/mL can be used.

Positive control for HL-60 cells

Dilute doxorubicin stock solution in cell culture medium to a final concentration of 50 μg/mL. Store at room temperature. Discard unused portion after experiment. Alternatively, calcium ionophore at the final concentration of 50 ng/mL can be used.

4.5 Negative Control

Use PBS as a negative control. Process it the same way as your study samples.

4.6 Heat-inactivated fetal bovine serum

Thaw a bottle of FBS at room temperature, or overnight at 2-8ºC and allow to equilibrate to room temperature. Incubate 30 minutes at 56ºC in a water bath, mixing every 5 minutes. Fifty (50) mL single use aliquots may be stored at 2-8ºC for up to one month or at a nominal temperature of -20ºC indefinitely.

4.7 Buffer A

Prepare Buffer A by dissolving NaCl to a final concentration of 150 mM and $CaCl_2$ to a final concentration of 6.6 mM in 20 mM HEPES, pH 7.4.

4.8 Preparation of coagulation controls (Coag N+ABN):

Reconstitute lyophilized control plasmas with 2 mL of distilled water. Let the solutions stand at room temperature 30 minutes prior to use. Mix thoroughly before use. Keep unused portion refrigerated and use within 48 h after reconstitution. These plasma samples are used as instrument controls.

4.9 Preparation of plasma for coagulation test:

Use freshly collected whole blood anti coagulated with Na-citrate (blue top vacutainers) within 1 hour after collection. Spin the blood 10 min at 2500 x g at 20-22°C; collect plasma and pool. Pooled plasma is stable for 8 h at RT. Do not refrigerate or freeze.

Analyze 2 duplicates of test-plasma in each coagulation assay, run one duplicate before nanoparticle-treated plasma samples and the second duplicate at the end of each run.

4.10 Vehicle Control

Vehicle control is the buffer or media used to formulate test nanomaterials. Common excipients used in nanoformulations are trehalose, sucrose, and albumin. However, other reagents and materials are also used alone or in combination.

Vehicle control should match formulation buffer of the test-nanomaterial by both composition and concentration. This control can be skipped if nanoparticles are stored in PBS.

4.11 Neoplastin reagent

This reagent is supplied as lyophilized powder along with reconstitution buffer. Reconstitute according to the manufacturer's instructions and use fresh or refrigerate and use within the time specified by the manufacturer.

5. Preparation of Study Samples

This assay requires 3 mL of nanoparticles or 1.5mL if using only PBMC or HL-60 model. The particles should be dissolved/resuspended in complete culture medium at a concentration 10 X of the highest tested concentration. The concentration is selected based on the plasma concentration of the nanoparticle at the intended therapeutic dose. For the purpose of this protocol this concentration is called "theoretical plasma concentration". Considerations for estimating theoretical plasma concentration were reviewed elsewhere (22) and are summarized in Box 1 below.

Box 1. Example Calculation of Nanoparticle concentration for In Vitro Test

In this example, we assume the mouse dose is known to be 123 mg/kg.

$$human\ dose = \frac{mouse\ dose}{12.3} = \frac{123\frac{mg}{kg}}{12.3} = 10\ mg/kg$$

Blood volume constitutes approximately 8% of body weight, (e.g. a 70 kg human has approximately 5.6 L (8% of 70) of blood). This allows us to get a very rough estimation of what the maximum blood concentration may be.

$$in\ vitro\ concentration_{human\ maxtrix} = \frac{human\ dose}{human\ blood\ volume} = \frac{70\ kg\ \times 10\frac{mg}{kg}}{5.6\ L}$$

$$= \frac{700\ mg}{5.6\ L} = 0.125\ mg/mL$$

The assay will evaluate 4 concentrations: 10 X (or when feasible 100X, 30X or 5X) of the theoretical plasma concentration, theoretical plasma concentration and two 1: 5 serial dilutions of the theoretical plasma concentration. When the intended therapeutic concentration is unknown, the highest final concentration is 1 mg/mL or the highest reasonably achievable concentration. For example, if the final theoretical plasma concentration to be tested is 0.2 mg/mL, then a stock of 20 mg/mL will be prepared and diluted 10-fold (2 mg/mL), followed by two 1: 5 serial dilutions (0.4 and 0.08 mg/mL). When 400 µL of each of these samples are combined in a culture plate well with 3.6mL of cells, the final concentrations of nanoparticles are 0.008, 0.04, 0.2, and 2 mg/mL. Each nanoparticle concentration is plated 2 times.

6. Cell Preparation

6.1 Isolation of human lymphocytes

6.1.1 Place freshly drawn blood anticoagulated with Li-heparin (green top vacutainers) into 15- or 50-mL conical centrifuge tubes; add an equal volume of room-temperature PBS and mix well.

6.1.2 Slowly layer the Ficoll-Paque solution underneath the blood/PBS mixture by placing the tip of the pipet containing the Ficoll-Paque at the bottom of the blood sample tube. Alternatively, blood/PBS mixture may be slowly layered over Ficoll-Paque solution. Use 3 mL of Ficoll-Paque solution per 4 mL of blood/PBS mixture.

Note: To maintain Ficoll-blood interface it is helpful to hold tube at 45° angle.

6.1.3 Centrifuge 30 minutes at 900 x g, 18-20°C, slow acceleration and without brake.

6.1.4 Using a sterile pipet, remove the upper layer containing plasma and platelets, and discard.

6.1.5 Using a fresh sterile pipet, transfer mononuclear cell layer into another centrifuge tube.

6.1.6 Wash cells by adding an excess of HBSS; centrifuge for 10 min at 400 x g, 18-20°C. The HBSS volume should be ~3 times the volume of the mononuclear layer.

Note: Typically, 4 mL of blood/PBS mixture results in ~2 mL of mononuclear layer and requires 6 mL of HBSS for the wash step.

6.1.7 Discard supernatant and repeat wash step once more.

6.1.8 Resuspend cells in complete RPMI 1640 medium. Count an aliquot of cells and determine viability using trypan blue exclusion.

6.1.9 If cell viability is ≥ 80%, dilute cells in complete culture media to the concentration of $3x10^6$ cells/mL and proceed to step 7.1.1.

6.2 Preparation of HL-60 Cells

6.2.1 Grow cells in complete culture media; avoid a cell density greater than $1x10^6$ cells/mL. On the day of experiment, count cells; if cell viability is ≥ 80%, concentrate cells in complete culture media to the concentration of $3x10^6$cells/mL and proceed to step 7.1.1.

7. Experimental Procedure

7.1 Cell treatment with nanoparticles and controls

7.1.1 Aliquot 3.6 mL of cell suspension into each well of a 6-well plate.

7.1.2 Add 400 µL of test nanoparticle, positive control and negative control to respective wells. Negative control for both cell models is PBS. Positive control for PBMC is LPS at final concentration of 1 µg/mL. Positive control for HL-60 cells is doxorubicin at a final concentration of 50 µg/mL. Ca2+ ionophore can also be used as a positive control. Prepare two wells for each sample. See Appendix for example plate map.

7.1.3 Incubate cells with nanoparticles and controls. Time of incubation is 5 hours for the HL-60 model and 24 h for PBMC.

7.1.4 At the end of the incubation time, remove cells from the incubator, transfer cells into 5mL falcon tubes, and wash cells two times with 1 mL of PBS. For each wash cycle spin cells at 400 x g for 5 min.

7.1.5 After last wash, reconstitute cell pellet in 1mL of Buffer A which usually results in cell concentration of $10x10^6$ cells/mL and transfer cells into 20mL scintillation vial or equivalent.

Note: any vial or tube can be used if it fits into coagulometer warming well

7.1.6 Keep cell suspensions at room temperature; place in the incubator (37˚C) 5-10 minutes prior to testing to warm up. The sample will then be transferred to the 37˚C chamber on the coagulometer when ready to start.

7.2 Determining plasma coagulation time

7.2.1 Set-up the instrument test parameters as shown below:

Max Time: 360 sec

Incubation Time: 120 sec

Single/Duplicate: Duplicate

Precision: 5%

Allow instrument to warm up to 5-10 minutes prior to use.

7.2.2 Prepare all reagents and cells, and warm them up to 37°C prior to use. Note that lyophilized reagents should be reconstituted at least 30 minutes prior to use. It is not advised to keep more than 10 cell samples at 37°C at one time.

7.2.3 Place cuvettes into A, B, C and D test rows on coagulometer (Note this protocol is based on semi-automatic STart4 coagulometer from Diagnostica Stago; if using a different instrument, please follow operation guidelines recommended by the instrument manufacturer).

7.2.4 Add one metal ball into each cuvette and allow to warm for at least 3 minutes before use.

7.2.5 Add 100 µL of control plasma (step 4.7) or test plasma (step 4.8) to a cuvette. Prepare 1 strip of cuvettes (1 strip, 4 wells) for each plasma sample.

7.2.6 Start timer for each of the test rows by pressing A, B, C or D buttons. Ten seconds before time is up, timer starts beeping. When this happens,

immediately transfer cuvettes to PIP row and press PIP button to activate pipettor.

7.2.7 When time is up, add 100 µL of Neoplastin reagent to control plasma samples or 100 µL of cell suspension from step 7.1.6 in lieu of coagulation activation reagent to corresponding cuvettes, and record coagulation time.

8. Calculations

8.1 A percent coefficient of variation should be calculated for each control or test sample according to the following formula:

%CV = (SD/Mean) x 100%

8.2 A percent procoagulant activity of a nanoparticle test sample is calculated according to the following formula:

$$\%PCA = (\text{mean time}_{\text{positive control sample}}/\text{time}_{\text{test sample}}) \times 100\%$$

9. Acceptance Criteria

9.1 %CV between replicates representing individual wells in the cuvette should be within 5%. This limit is stored in the instrument setting. When %CV is greater than 5, you will see a * mark on the printout.

9.2 % CV between replicates of test plasma samples should be within 25%.

9.3 If two duplicates of the same study sample demonstrated results >25% different, this test sample should be re-analyzed.

9.4 Positive control is considered positive if coagulation time in this sample is less than 360 sec. Coagulation time induced by HL-60 cells treated with doxorubicin and that induced by PBMC treated with LPS may vary from passage to passage and from donor to donor, respectively. Typical coagulation times observed in our lab with HL-60 cells treated with 50 µg/mL doxorubicin is 60-90 seconds. Typical coagulation times observed in our lab with PBMC treated with 1 µg/mL LPS is 200-300 seconds; that induced by ionophore is about 150 seconds.

10. References

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11. Abbreviations

APL	acute promyelocytic leukemia
CV	coefficient of variation
DIC	disseminated intravascular coagulation

FBS	fetal bovine serum
HBSS	Hank's balanced salt solution
LPS	lipopolysaccharide
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCA	procoagulant activity
RPMI	Roswell Park Memorial Institute
RT	room temperature
SD	standard deviation
U	units

12. Appendix

Example Culture Plate Maps

	1	2	3
A	Medium (baseline)	NC	PC
B	Medium (baseline)	NC	PC

	1	2	3
A	VC	TS 1 1.0 mg/mL	TS 1 0.2 mg/mL
B	VC	TS 1 1.0 mg/mL	TS 1 0.2 mg/mL

	1	2	3
A	TS 1 0.04 mg/mL	TS 1 0.008 mg/mL	
B	TS 1 0.04 mg/mL	TS 1 0.008 mg/mL	