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Research paper

EdU incorporation is an alternative non-radioactive assay to [^{3}H]thymidine uptake for in vitro measurement of mice T-cell proliferations

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ABSTRACT

Rationale: T lymphocyte proliferations can be measured by [^{3}H]thymidine incorporation. However, many labs avoid this technique because of the need to use radioactive substrates. In addition, [^{3}H]thymidine incorporation method does not permit simultaneous characterization of the proliferating cells. We developed the 5-ethynyl-2′-deoxyuridine (EdU) and Cu(I)-catalyzed cycloaddition "click" reaction assay to measure T-cell responses by flow cytometry.
Methods: Spleen cells from normal, immune-deficient purine nucleoside phosphorylase (PNP) defective (PNP−/−) mice or PNP−/− mice with partial immune reconstitution were stimulated with anti-CD3 antibodies. The correlation (r) between [^{3}H]thymidine and EdU incorporations into stimulated T cells was measured and the stimulation index (SI), the ratio between stimulated and non-stimulated cells, was calculated. Flow cytometry was used to characterize the proliferating cells.
Results: EdU and [^{3}H]thymidine incorporation into normal spleen cells were strongly correlated ($r = 0.89$). Following stimulation, EdU incorporation into spleen cells from normal and immune-reconstituted PNP−/− mice was significantly increased compared to PNP−/− immune-deficient mice. Immune-deficient PNP−/− mice had increased [^{3}H]thymidine and EdU incorporation into non-stimulated spleen cells, indicative of spontaneous proliferation. Analysis of EdU incorporation showed that the increased proliferation was due primarily to cells expressing CD3, CD4 and IgM.
Conclusion: EdU-Click technology accurately measures proliferation of murine T lymphocyte and can be used as an alternative to [^{3}H]thymidine assays. The EdU-Click technology also allows identification of proliferating cells.

1. Introduction

Evaluations of T-cell proliferations are critical for understanding the immune system (Taghon et al., 2009) and managing patients with various T-lineage immune abnormalities (Stone et al., 2009; Grunebaum et al., 2006). DNA synthesis, required for generation of new cells, is essentially synonymous with cell proliferation (Sawada et al., 1995; Macallan et al., 1998). DNA synthesis, commonly measured by the incorporation of radioisotope-labeled nucleosides such as [^{3}H]thymidine, has become the "gold standard" method for assessing T-cell responses (Waldman et al., 1991). Some researchers also calculate the "stimulation index" (SI), the ratio between [^{3}H]thymidine incorporation into stimulated cells and non-stimulated cells, to compensate for variables including different numbers and concentration of cells in initial cultures or contamination with non-lymphoid cells (Grunebaum et al., 2006; Goodell et al., 2007; Stone et al.,

Abbreviations: EdU, 5-ethynyl-2′-deoxyuridine; BrdU, 5-bromo-2-deoxyuridine; PNP, purine nucleoside phosphorylase; PBMC, peripheral blood mononuclear cells; cpm, counts per minute.

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2009). The [^{3}H]Thymidine method requires radioactive reagents and cumbersome detection devices, therefore many clinical and research laboratories avoid this technique. Moreover, the [^{3}H]thymidine detection method does not permit simultaneous characterization of the proliferating cells.

Bromodeoxyuridine (BrdU), a nucleoside analogue that incorporates into genomic DNA, was developed as a non-radioactive alternative to detect cell proliferations. The BrdU method has gained popularity since the ability to measure BrdU with fluorescent tagged antibodies (Gratzner, 1982). However, BrdU detection requires subjecting the samples to strong denaturing conditions that modify cellular epitopes, thereby hindering analysis of the cell's antigens and causing variability in the staining intensity (Rakic, 2002).

Recently, another non-radioactive small thymidine analogue, 5-ethynyl-2′-deoxyuridine (EdU), was developed by replacing the methyl group at position 5 of deoxyuridine with an alkyne group. EdU was shown to readily incorporate into DNA of replicating cells including NIH 3T3 cells, HeLa cells, Xenopus egg extracts (Salic and Mitchison, 2008; Chehrehasa et al, 2009) and human T-cell leukemia Jurkat cell line (Buck et al., 2008). Importantly, the alkyne group of EdU can react with fluorescent azides in a Cu(I)-catalyzed [3 + 2] cycloaddition reaction known as "click" (illustrated in Fig. 1A), enabling detection of EdU incorporation into cells by fluorescent microscopy or fluorescence activated cell sorter (FACS). Few groups have since used EdU incorporation to assess proliferations of ovarian cancer, leukemia and osteosarcoma cell lines (Cappella et al., 2008) and visualize cells in murine intestines and brain sections (Salic and Mitchison, 2008; Chehrehasa et al, 2009).

We hypothesized that the EdU-Click technology could also detect T-cells proliferations and distinguish between T lymphocytes from normal, T-cell immune-deficient or partially immune-reconstituted mice. In addition, we reasoned that the EdU-Click technology would allow further analysis into the nature of the proliferating cells. To explore these possibilities, we used the purine nucleoside phosphorylase (PNP) deficiency murine model. Defective PNP function causes accumulation of toxic metabolites that lead to severe T-cell immunodeficiency and autoimmunity (Markert, 1991). PNP-deficient (PNP−/−) mice display increased thymocyte apoptosis and reduced responses of peripheral T cells to stimulation (Arpaia et al., 2000; Toro and Grunebaum, 2006). We previously showed that treating PNP−/− mice with repeated injections of PNP fused to the HIV TAT protein transduction domain (PTD–PNP) or transplanting the mice with PNP-deficient bone marrow cells transduced with lentivirus vectors expressing PNP (lentiPNP) may temporarily improve T-lineage development and T-cell proliferations (Toro and Grunebaum, 2006; Liao et al., 2008).

We demonstrate here that EdU-Click technique reliably detects the proliferation of T cells, similar to [^{3}H]thymidine. We also show that EdU-Click distinguishes between T lymphocytes from normal mice, PNP−/− mice with partial immune reconstitution and immune-deficient PNP−/− mice. Moreover, the EdU-Click technique allows detailed analysis of the proliferating cells.

2. Materials and methods

2.1. Cells

PNP−/− mice, in whom the catalytic domain of the PNP enzyme was disrupted, were described previously (Arpaia et al., 2000). Partial immune reconstitution was achieved by treating PNP−/− mice with repeated PTD–PNP injections and by transplanting them with bone marrow cells harvested from littermate PNP−/− mice, that were transduced with lentiPNP, as previously described (Toro and Grunebaum, 2006; Liao et al., 2008). Spleen cells were collected from PNP−/− mice with no or partial immune reconstitution and from normal littermates, filtered through 40 um nylon cell strainers (BD Falcon, Bedford, MA, USA) followed by red blood cells lysis with Ammonium Chloride (Stemcell technologies, Vancouver, BC, Canada). The remaining cells were maintained in RPMI with 10% Fetal Calf Serum (Invitrogen, Burlington, ON, Canada) penicillin and streptomycin. The Research Ethics Board at The Hospital for Sick Children, Toronto, ON, Canada, approved all procedures.

2.2. EdU incorporation and Click-iT ™ reaction

For EdU incorporation experiments, cells (1×10^6/ml) were stimulated with mouse anti-CD3 antibodies (10 μg/ml, BD bioscience) for 48–72 h in 24-well plates. EdU (from the Click-iT™ EdU Flow Cytometry Assay Kit, Invitrogen™, OR, USA) was added at a 50 μM final concentration 24 h before harvesting the cells. For the Click reaction, cells were collected into 3 ml of PBS containing 1% BSA, centrifuged and fixed with 100 μl of 4% paraformaldehyde for 15 min. Cells were washed again and incubated with 100 μl of saponin-based permeabilization buffer for 15 min. After additional washing, cells were incubated with 500 μl Click-iT reaction buffer for 1 h and washed again with 3 ml permeabilization buffer. All procedures were performed according to the manufacturer's instructions except for some experiments where the reaction buffer was reduced from 500 μl to 250 μl and Copper (II) Sulfate was obtained from an alternative source (Sigma-Aldrich, Oakville, ON, Canada).

2.3. Flow cytometry

Flow cytometry was performed using a FACSCaliber Flow Cytometer (BD Biosciences, Mississauga, ON, Canada) and data was analyzed with the FlowJo analysis software (Tree Star, Inc., Ashland, OR, USA). Analysis of 2×10^4 total events was performed after exclusion of dead cells by FSC/SSC gating. Fluorescence I channel was used to detect cells (events) that incorporated EdU. In some experiments, following EdU incorporation, spleen cells were labeled with biotin conjugated anti-mouse antibodies to CD3, CD4, CD8, CD11b, NK1, Mac3, Gr1 and IgM (eBioscience, San Diego, CA, USA). Following washes, APC-conjugated anti-biotin antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany) were added. After fixation and permeabilization of the cells, the Click reaction was continued as described above.