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Effects of EdU labeling on mesenchymal stem cells

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Abstract

Background. Thymidine analog 5-ethynyl-2-deoxyuridine (EdU) has recently been used for tracking mesenchymal stem cells (MSCs). In the present study, we tested whether EdU was cytotoxic and whether it interfered with differentiation, cytokine secretion and migration of MSCs. *Methods.* EdU labeling was performed by incubating adipose-derived stem cells (ADSCs) with 10^{-8} mol/L of EdU for 48 h. Incorporation of EdU was detected by reaction with azide-conjugated Alexa594. The labeled and unlabeled ADSCs were compared for proliferation and apoptosis as determined by CellTiter and comet assays, respectively. They were also compared for neuron-like and endothelial differentiation as determined by morphology, marker expression and function. Comparison of their secreted cytokine profile was performed by cytokine antibody array. Comparison of their response to homing factor SDF-1 was performed by migration assay. *Results.* EdU was incorporated into the nucleus in approximately 70% of ADSCs. No significant differences in proliferation and apoptosis rates were observed between EdU-labeled and unlabeled ADSCs. Isobutylmethylxanthine induced both EdU-labeled and unlabeled ADSCs to assume a neuron-like morphology and to express β-III tubulin. Endothelial growth medium-2 (EGM2) induced endothelial differentiation in both EdU-labeled and unlabeled ADSCs, including the ability to uptake low-density lipoprotein and to form capillary-like structures as well as the expression of vWF, eNOS and CD31. EdU-labeled and unlabeled ADSCs exhibited identical secreted cytokine profile and identical migratory response to SDF-1. *Discussion.* At the recommended dosage of 10^{-8} mol/L, EdU is non-toxic to ADSCs. EdU label did not interfere with differentiation, cytokine secretion or migratory response to SDF-1 by ADSCs.

Key Words: *adipose-derived stem cells, cell labeling, cytokine expression, differentiation, EdU, mesenchymal stem cells, migration*

Introduction

Thymidine analogs have been used extensively for the analysis of cellular DNA synthesis, tracking of transplanted cells and identification of tissue-resident stem cells (1–4). The importance of these thymidine analogs in biomedical research is best exemplified by the utilization of 5-bromo-2-deoxyuridine (BrdU) in more than 20,000 peer-reviewed studies (1). However, the histological detection of BrdU requires harsh conditions that affect cellular structure and protein anti-genicity. In addition, the resulting histological images can be difficult to assess because of low signal-versus-noise ratio. As such, in 2008, Salic and Mitchison (5) introduced a new thymidine analog, 5-ethynyl-2′-deoxyuridine (EdU), which can be easily detected without affecting cellular structure or protein anti-genicity. In 2009, we reported for the first time the use of EdU for the labeling and tracking of mesenchymal stem cells (MSCs), specifically, adipose-derived stem cells (ADSCs) (2). We have since used this method to track transplanted ADSCs in several preclinical studies, for example, in a rat model of stress urinary incontinence (3). We have also used it for the detection of potential tissue-resident stem cells by means of the "label-retaining cell" strategy (4). In studies that examined possible cellular differentiation of transplanted ADSCs, we occasionally observed smooth muscle or endothelial differentiation, based on the co-localization of the EdU label with specific cell lineage markers (3,6,7). However, such co-localization occurred only rarely, and therefore we proposed that therapeutic effects of ADSCs might derive from its paracrine action rather than cellular differentiation (3,8,9). However, it has been shown that BrdU was cytotoxic and inhibited cell differentiation and migration (10,11). Thus, it is possible that the infrequent cellular differentiation of transplanted ADSCs was a consequence of EdU labeling.

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On the other hand, it has also been shown that BrdU labeling did not have adverse effects on ADSCs (12). Therefore, amid these conflicting reports on the adverse effects of BrdU (or lack of), the present study aimed at testing whether EdU labeling affects cell proliferation, apoptosis, cytokine secretion, differentiation and migration.

Methods

EdU labeling of ADSCs

Isolation and culture of rat ADSCs have been described previously (3). Cells at the fourth passage were used in the present study. For EdU labeling, 1×10^5 cells were seeded in each well of a six-well plate in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Twenty-four h later, EdU (Invitrogen, Carlsbad, CA, USA) was added to the medium at 10^{-8} mol/L. Another 48 h later, cells were fixed for EdU staining or for further tests. For EdU staining, the cells were fixed with methanol, washed twice with phosphate-buffered saline (PBS), incubated in 3% bovine serum albumin in PBS and then incubated in 0.5% Triton X-100 in PBS for 20 min at room temperature. The cells were then incubated with freshly made Click-iT reaction cocktail, which contained azide-conjugated Alexa594 (Invitrogen), for 30 min at room temperature in the dark. The cells were further stained with 4′,6-diamidino-2-phenylindole (DAPI, for nuclear staining, 1 μg/mL, Sigma-Aldrich, St. Louis, MO, USA) and then mounted in standard mounting media. The stained cells were examined with a Nikon Eclipse E600 fluorescence microscope and photographed with a Retiga 1300 Q-imaging camera. For determination of the percentage of EdU-positive cells, the number of red-fluorescent (Alexa594-stained) cells was divided by the number of blue-fluorescent (DAPI-stained) cells. The experiment was performed in triplicate. Data presented in Results are the average of three independent experiments.

Proliferation assay

EdU-labeled or unlabeled ADSCs were seeded into a 96-well plate at 2000 cells per well in DMEM with 10% FBS and incubated at 37°C. At 0, 24, 48, 72 and 96 h, 10 μL of CellTiter-96 reagent (Promega Inc., Madison, WI, USA) was added to each well. After 1 h of further incubation at 37°C, the cells were scanned in a plate reader (Molecular Devices Corp., Sunnyvale, CA, USA) at 490-nm absorbance. All assays were performed in triplicate. All data presented in Results are the average of three independent experiments.

Apoptosis assay

Cellular apoptosis was analyzed by the CometAssay Electrophoresis Systems (Trevigen, Inc., Gaithersburg, MD, USA). Briefly, 50 μL (5000 cells) of EdU-labeled or unlabeled ADSCs in PBS were mixed with 500 μL of molten low-melting agarose at 37°C. An aliquot of 75 μL of the mixture was immediately pipetted and spread evenly onto a CometSlide (Trevigen). The slide was then placed in a refrigerator for 10−30 min and immersed in pre-chilled lysis solution for another 30−60 min. Afterward, the slide was immersed in freshly prepared alkaline solution for 20−60 min at room temperature in the dark. The slide was then washed with Tris-borate-EDTA (TBE) buffer twice for 5 min each and electrophoresed in TBE at 1 v/cm for 10 min. Afterward, the slide was dipped in 70% ethanol for 5 min and air-dried (until the agarose became dry). An aliquot of 50 μL of diluted SYBR green I (Trevigen) was then added onto each well of the dried agarose. The slide was then observed with a Nikon Eclipse E600 fluorescence microscope (Nikon Instruments Inc., Melville, NY, USA) and photographed with a Retiga 1300 camera (QImaging, Burnaby, BC, Canada). For determination of the percentage of apoptotic cells, the number of cells with comet tail (indication of apoptosis) was divided by the number of all cells. The experiment was performed in triplicate. Data presented in Results are the average of three independent experiments.

In vitro neuronal differentiation

ADSC neuron-like differentiation was performed as previously described (13). Briefly, EdU-labeled or unlabeled ADSCs were seeded into a six-well plate at 5×10^5 cells per well in DMEM with 10% FBS. The next day, the cells were washed three times with PBS and the medium changed to DMEM supplemented with 5×10^{-8} mol/L isobutylmethylxanthine (IBMX) (Sigma-Aldrich). One hour later, the cells were stained for the presence of EdU and for neuron-specific marker β-III tubulin (14) (using anti−β-III tubulin antibody from Abcam, Inc., Cambridge, MA, USA), followed by nuclear stain with DAPI.

In vitro endothelial differentiation

ADSC endothelial differentiation was performed as previously described (7). Briefly, EdU-labeled or unlabeled ADSCs were seeded into a six-well plate at 5×10^5 cells per well in DMEM with 10% FBS. The next day, the culture medium was replaced with EGM2 (Lonza Biologics Inc., Portsmouth, NH, USA), and the cells further incubated for 7 days with the medium replenished every 3 days. The cells were then analyzed for endothelial marker expression and