Biochimica et Biophysica Acta 1853 (2015) 27–40

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

An optical labeling-based proliferation assay system reveals the paracrine effect of interleukin-6 in breast cancer

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ARTICLE INFO

Article history:
Received 20 June 2014
Received in revised form 25 September 2014
Accepted 1 October 2014
Available online 12 October 2014

Keywords:
Breast cancer
Interleukin-6
Optical labeling
Paracrine effect
Proliferation
The OPA system

ABSTRACT

Proliferation analysis is one of the basic approaches to characterize various cell types. In conventional cell proliferation assays, the same sample cannot be observed over time, nor can a specific group within a heterogeneous population of cells, for example, cancerous cells, be analyzed separately. To overcome these limitations, we established an optical labeling-based proliferation assay system with the Kaede protein, whose fluorescence can be irreversibly photoconverted from green to red by irradiation. After a single non-toxic photoconversion event, the intensity of red fluorescence in each cell is reduced by cell division. From this, we developed a simple method to quantify cell proliferation by monitoring reduction of red fluorescence over time. This study shows that the optical labeling-based proliferation assay is a viable novel method to analyze cell proliferation, and could enhance our understanding of mechanisms regulating cell proliferation machinery. We used this newly established system to analyze the functions of secreted interleukin-6 (IL-6) in cancer cell proliferation, which had not been fully characterized. Reduction in proliferation was observed following IL-6 knockdown. However, after co-culturing with IL-6-expressing cells, the proliferation of Kaede-labeled IL-6-knockdown cells was restored. These data indicate that in basal-like breast cancer cells, IL-6 exhibits a paracrine effect to positively regulate cell proliferation. Our results thus demonstrate that cancer cells can secrete signaling molecules, such as IL-6, to support the proliferation of other cancer cells.

1. Introduction

The cell proliferation machinery orchestrates events including DNA replication, duplication of cellular components, disappearance of the nuclear membrane, chromosome segregation, reconstruction of the nuclear membrane and cytoplasmic division. Although a significant number of studies have identified various factors that regulate cell proliferation, we still have much to learn about how these processes are regulated, particularly in transformed cells.

Cancer cells are highly proliferative as a consequence of the up-regulation of cell cycle activators [1,2], the activation of signaling pathways [1,3], mutations in genes controlling the cell cycle [1,4], genomic amplification [5], and epigenetic changes [6]. The majority of cancer cells actively proliferate in culture, and cancer cell cultures are useful models for the study of cell proliferation.

Breast cancer cells develop in mammary microenvironments, arising from the mammary epithelium. However, molecular and cellular characteristics, including proliferative ability, vary among breast cancer cells [7,8]. Breast cancers are classified into subtypes according to differences in the expression of various markers [9–11]. The existence of various types of breast cancer originating from an identical cellular background suggests that use of these cell lines can enable the study of a variety of potential mechanisms used to activate the proliferation machinery, without having to consider differences in cellular origin and environmental stimuli.

Conventional methods of analyzing cell proliferation can be grouped into three categories. The first method is counting the number of cells. Cells are harvested at specified time points, and proliferation is quantified by counting the number of cells following trypan blue staining. An alternative is to measure the activity of succinate–tetrazolium reductase, which is active in living cells and inactive in dead cells. The second analytic method is incubating proliferating cells with thymidine analogs, such as tritiated thymidine, bromodeoxyuridine (BrdU), and ethynyldeoxyuridine. Cells that have proliferated are identified by the

Abbreviations: BrdU, bromodeoxyuridine; hpi, hours post-irradiation; IL-6, interleukin-6; MMC, mitomycin C; STAT3, signal transduction and activator of transcription 3; The OPA system, the optical labeling-based proliferation assay system

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http://dx.doi.org/10.1016/j.bbamcr.2014.10.004
0167-4889/

presence of thymidine analogs post-incubation, which are incorporated into the genome during S-phase. The third method is to detect proliferation markers by immunostaining. Cells are fixed and stained with specific antibodies that target, for instance, the cell proliferation marker Ki-67, the S-phase marker PCNA, or the M-phase marker phosphohistone H3.

Although these conventional methods are widely used, they have a number of limitations. The data obtained by these methods consist of sample values harvested from different cultures for each time point and are not a continuous observation of the same culture. These methods are unsuitable for analyzing the proliferative ability of a particular cell type or group of interest in a heterogeneous population. While staining for proliferation markers can detect proliferating cells in a specific group, these results are only snapshots of growth. Therefore, a novel method complementary to these conventional proliferation assays is sorely needed.

Extracellular signaling molecules can stimulate proliferation. Interleukin-6 (IL-6) is a secreted protein encoded by the *IL6* gene. IL-6 forms a heterocomplex with the IL-6 receptor and glycoprotein 130 on the cytoplasmic membrane, which activates the Janus kinase/signal transduction and activator of transcription 3 (STAT3) intracellular signaling pathway [12,13]. IL-6 secretion is high in basal-like breast cancer, a cancer subtype that possesses high proliferative and metastatic ability [14–16]. IL-6 secreted by cancer cells has been suggested to have paracrine effects in this type of breast cancer. In terms of cell proliferation, however, the involvement of IL-6 is controversial (reviewed in [17]), as administration of IL-6 has been reported to variously inhibit, enhance, or have no effect on cell proliferation [17]. Further study with novel techniques will provide a new insight into the function of IL-6 in cancer cell proliferation.

This study describes the design of an optical labeling-based proliferation assay system (the OPA system) that utilizes a photoconvertible fluorescent protein. As the fluorescence emission wavelengths of photoconvertible proteins are changed by irradiation, we can therefore label particular cells by irradiation (optical labeling) [18,19]. In biology research, optical labeling is known as a technique that allows researchers to trace the behavior of a single cell, as well as analyze the dynamics of a group of cells, such as group migration, distribution, and growth. Different from these approaches, the OPA system uses optical labeling to quantify cell proliferation based on the reduction in photoconverted fluorescent protein molecules per cell following cytoplasmic division.

The Kaede protein is a photoconvertible fluorescent protein whose color irreversibly changes from green to red after irradiation with short wavelength light [20]. In this study, we used the Kaede protein in the OPA system. We detected reductions in the fluorescence of photoconverted Kaede by simply taking pictures and measuring its intensity in living cells, thus successfully quantifying proliferation. The OPA system enables us to perform observations of cell proliferation in the same culture over time. Furthermore, we can analyze the proliferative ability of a specific group of cells in co-culture experiments by labeling the group of interest with Kaede. This study shows that the OPA system facilitates the understanding of the molecular mechanisms of cell proliferation. Using the OPA system, we examined the effect of IL-6 knockdown in breast cancer cell lines. We also analyzed the proliferation of IL-6-knockdown cells in co-culture experiments with IL-6-producing cells. Our results show the function of cancer-secreted IL-6 in cancer cell proliferation.

2. Materials and methods

2.1. Cell culture

Basal-like breast cancer cell lines Hs578T and MDA-MB-231, and luminal breast cancer cell line MCF-7 were obtained from the American Type Culture Collection (Manassas, VA, USA). Hs578T and MDA-MB-231 cells were maintained with Dulbecco's Modified Eagle Medium (DMEM) (Sigma, R8758, St. Louis, MO, USA) with 10% fetal bovine serum (FBS). MCF-7 cells were cultured with RPMI-1640 (Sigma, D5796) containing 10% FBS and 1 nM estradiol (Sigma, E2758). Lenti-X 293T cells (Takara Bio, 632180, Otsu, Japan), used to produce lentiviral particles, were maintained with DMEM containing 10% FBS. To obtain Kaede-expressing cell lines and shRNA-expressing cells, drug selection was performed. We used 10 μg/mL blasticidin for Kaede-expressing cells, and 1 μg/mL puromycin for shRNA-expressing cells.

To analyze proliferation using the OPA system, cells were cultured with phenol red-free media to reduce the background fluorescence: i.e., phenol red-free DMEM (Wako, 044-32955, Osaka, Japan) with 10% FBS for Hs578T and MDA-MB-231 cells, and phenol red-free RPMI-1640 (Life Technologies, 11835-030, Carlsbad, CA, USA) with 10% FBS and 1 nM estradiol for MCF-7 cells.

Mitomycin C (MMC) was used to pharmacologically inhibit proliferation. MMC was purchased from Wako (133-15931). Control groups were treated with the vehicle, i.e., 10% ethanol in ethylene glycol.

To measure the red intensity of the entire well, we used a microplate reader, Spectra Max Gemini EM (Molecular Devices, Sunnyvale, CA, USA). In 1 day prior to assay, 1×10^4 cells per well were plated in a 96-well plate. Red fluorescence was excited with 544 nm light, and detected as 590 nm light. Data were collected with Soft Max Pro 5 software (Molecular Devices). Values were normalized to the non-photoconverted control.

2.2. Vector construction

To introduce Kaede and shRNA expression, we used lentiviral vectors. For Kaede expression, we used pLenti 6.3 vector (Life Technologies, V533-06), which has a CMV promoter. The *Kaede* gene was purchased from Medical and Biological Laboratories (AM-V0011, Nagoya, Japan). We added a synthesized nuclear localization signal (NLS)-FLAG sequence to the 3′ region of the *Kaede* gene in order to construct a Kaede-NLS-FLAG fusion gene. The Kaede-NLS-FLAG fusion gene was inserted downstream of the CMV promoter of the pLenti 6.3 vector.

For shRNA-mediated gene silencing, we used a pLKO.1 vector (Addgene, 8453), which expresses the inserted shRNA sequence by human U6 promoter. The synthesized shRNA template was inserted between the *Age*I *Eco*RI sites of the pLKO.1 vector. The shGFP and shBMI1 sequences have been described previously [21]. The shIL6 sequence used was validated by a previous study [15].

2.3. Immunostaining

Immunostaining was performed to detect Kaede, pSTAT3 and STAT3 proteins, and cyclobutane pyrimidine dimers in fixed cells. The procedure has been described previously [21]. Cells were fixed with 4% paraformaldehyde for 15 min at room temperature. Permeabilization was performed with 10 μg/mL proteinase K (Wako, 169-21041) in phosphate-buffered saline (PBS) on ice for 2 min. Primary antibodies used were mouse anti-FLAG M2 antibody (Sigma, F1804, 1/300 dilution), mouse anti-cyclobutane pyrimidine dimer antibody TDM-2 (Cosmo Bio, NMDND001, Tokyo, Japan, 1/1500 dilution), rabbit anti-phospho-STAT3 Tyr705 (D3A7) (Cell Signaling technology, X9145, Danvers, MA, USA, 1/100 dilution) and rabbit anti-STAT3 antibody (D3Z2G) (Cell Signaling Technology, 12640, 1/300 dilution). To detect primary antibody binding via fluorescence microscopy, we used secondary antibodies, goat anti-mouse IgG antibody conjugated to Alexa Fluor 488 (Life Technologies, A11001, 1/1000 dilution), and goat anti-rabbit IgG antibody conjugated to Alexa Fluor 546 (Life Technologies, A11010, 1/1000 dilution). Counter staining was performed with Hoechst 33342 (Dojindo, 346-07951, Kamimashiki, Japan, 1/1000 dilution).