Journal of Bioscience and Bioengineering
VOL. 122 No. 3, 357–363, 2016

ELSEVIER

www.elsevier.com/locate/jbiosc

Differentiation signalobody: Demonstration of antigen-dependent osteoclast differentiation from a progenitor cell line

Hideto Nakabayashi, Saeko Aoyama, Masahiro Kawahara,* and Teruyuki Nagamune

Department of Chemistry and Biotechnology, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

Received 18 December 2015; accepted 19 February 2016
Available online 12 March 2016

A "cytokine-less" *in vitro* differentiation method would be promising for cost-effective mass production of cells used for regenerative medicine. In this study, we developed a differentiation signalobody S-RANK, in which the extracellular domain of receptor activator of nuclear factor kappa-B (RANK) is replaced with a single-chain variable fragment (scFv) to attain signaling in response to an inexpensive antigen. A murine macrophage cell line RAW264, which is known to differentiate into an osteoclast by RANK ligand (RANKL), was lentivirally transduced with S-RANK. When the resultant cells were cultured with a specific antigen, the cells differentiated into multinucleated tartrate-resistant acid phosphatase-positive osteoclasts. The differentiation efficiency was almost comparable to those induced by RANKL. In addition, the signaling analysis demonstrated that nuclear factor kappa-B and mitogen-activated protein kinase signaling pathways, which are the major signaling pathways downstream of wild-type RANK, were also activated by S-RANK. These results demonstrate that S-RANK sufficiently mimics signal transduction of wild-type RANK. Differentiation signalobodies may be applied for controlling differentiation of other cell types by using appropriate signaling domains.

[**Key words:** Cell differentiation; Osteoclast; Signalobody; Chimeric protein; Signal transduction]

Adult tissue stem cells and progenitor cells are mainly used for current cell-based therapies, in which these undifferentiated cells are administered directly *in vivo* to expect spontaneous differentiation into desired cell lineages at a targeted tissue. For example, bone marrow transplantation is recognized as an effective therapy for hematopoietic diseases such as leukemia, because hematopoietic stem cells (HSCs) in the transplanted cells are engrafted in the patient and differentiate into all kinds of hematopoietic cells (1). Mesenchymal stem cells (MSCs), which can differentiate into multiple cell lineages including osteoblasts (2), cardiomyocytes (3), adipocytes (4), and hepatocytes (5), have also been used for transplantation to reconstitute injured organs. However, the therapeutic effects were limited because of low frequency of cellular integration into the organs *in vivo* (6,7). To circumvent this issue, one possible solution is to produce a sufficient number of differentiated cells which could integrate into injured organs. To realize this, methods for mass production of differentiated cells *in vitro* are necessary. Recently, human embryonic stem (ES) cells (8) and induced pluripotent stem (iPS) cells (9,10) have been studied intensively as an alternative stem cell source because they have both self-renewal ability and pluripotency. Such characteristics facilitate production of a large number of differentiated cells through an *ex vivo* culture, opening up a new avenue toward effective cell-based therapies.

For clinical applications of cell-based therapies, it is necessary to guarantee the safety of the cells administered *in vivo*. To this end, the *ex vivo* culture of cells must be performed in a xeno-free condition (11). Conventionally, animal-derived sera and feeder cells, which supply growth factors and adhesion factors, were usually used for enhancing cell growth and differentiation. Since the conditions are not xeno-free, feeder- and serum-free differentiation methods have been developed. To date, several xeno-free ES/iPS cell culture media have been developed and used in cell differentiation studies, where cytokines need to be supplemented as growth and differentiation factors (12,13).

Cell-based therapy demands a large number of cells. For example, approximately 10^{12} platelets are required for reconstituting those in a whole body (14). To obtain that number of cells, cell culture will be so costly due to the addition of expensive cytokines that are necessary for cell differentiation. Therefore, development of a "cytokine-less" differentiation method is important for realizing cost-effective cell-based therapies.

Previous reports have demonstrated that cell differentiation can be induced by replacing cytokines with small molecules that inhibit or activate cellular signaling, or by introducing transcription factors named master regulators into cells. In the former method, it is necessary to screen small molecules that induce cells to differentiate into a specific lineage from a large library. While small molecules including IWR-1, (-)-Indolactam V, and IDE1/2 were surely discovered as effective differentiation inducers (15–17), such molecules will not always be discovered for all differentiation lineages. In the latter method, it is necessary to discover master regulators which can induce cell differentiation into a specific lineage of interest (18,19). Both methods more or less require a screening procedure to find candidates that direct cells toward a specific differentiation lineage.

* Corresponding author. Tel.: +81 3 5841 7290; fax: +81 3 5841 8657.
E-mail address: kawahara@bio.t.u-tokyo.ac.jp (M. Kawahara).

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2016.02.010

To solve this problem, we aimed to develop a method that easily mimics cytokine effects for inducing cell differentiation. Cytokines bind to the extracellular domain of their receptors on the cell surface and induce oligomerization of the receptors. After the oligomerization of the receptors, the cytoplasmic domain of the receptors recruits signaling molecules to initiate signal transduction. To mimic such an activation mechanism of cytokine receptors, we developed chimeric receptors in which the extracellular domain of cytokine receptors is replaced with a single-chain variable fragment (scFv) to alter their ligand specificity. By using this strategy, we have developed a series of antibody/receptor chimeras named signalobodies that can respond to a specific antigen. To date, cell proliferation (20–22), migration (23,24), and death (25) were successfully mimicked by signalobodies; however we have never tested signalobodies for mimicking cell differentiation.

Here, we focused on receptor activator of nuclear factor kappa-B (RANK). RANK, which belongs to the tumor necrosis factor receptor superfamily, is known to be expressed ubiquitously in various tissues (26). The studies using RANK knockout mice demonstrated that RANK plays an essential role in osteoclastogenesis (27), lymph node formation (28), and mammary gland development (29). Osteoclasts execute bone resorption and play an essential role in bone metabolism along with osteoblasts, which are bone-forming cells. Imbalance of bone resorption and formation causes several bone diseases such as osteoporosis. The RANK ligand (RANKL) is a type 2 transmembrane protein and forms a trimer (30,31). Stromal cells and osteoblasts express RANKL, which binds to RANK expressed on osteoclasts and induces RANK trimerization. The RANK trimer recruits tumor necrosis factor receptor-associated factors (TRAFs) and activates downstream signaling molecules including NF-κB, c-Jun N-terminal kinase (JNK), and p38 (26,32). These signaling events finally induce the expression and/or activation of transcription factors including c-Fos and NFATc1 which are related to osteoclastogenesis (33,34). RAW264 is a murine macrophage cell line which can differentiate into an osteoclast with RANKL stimulation. This cell line is often used for the studies of osteoclastogenesis and functional characterization of RANK (34,35). To demonstrate a proof of concept that signalobodies could be applied to cell differentiation, here a signalobody based on RANK was constructed and expressed in RAW264 cells. We investigated whether a specific antigen could induce osteoclast differentiation through the signalobody.

MATERIALS AND METHODS

Vector construction A plasmid, pL-SIN-EF1α-EGFP, was a gift from Dr. James Ellis (Addgene plasmid #21320 (36)). An internal ribosomal entry site (IRES)-puromycin resistance gene cassette and the sequence of the chimeric receptor consisting of an HA tag, an anti-fluorescein (FL) scFv, the extracellular D2 domain of human erythropoietin receptor (EpoR), and a stuffer were introduced into the vector instead of the EGFP sequence, resulting in pL-SIN-EF1α-HA-scFv-D2-stuffer-IRES-Puro^R. The extracellular domain of the chimeric receptor was the same of pGCDNsam-HA-S-Mpl(WT)-I/E (22). To replace the stuffer with the transmembrane and intracellular domains of human RANK, pL-SIN-EF1α-HA-scFv-D2-stuffer-IRES-Puro^R was linearized with PCR using two primers (Forward, 5′-TAGGATCCGCCCCTCTCCCT-3′; Reverse, 5′-GATATCGGGGTCCAGGTCGC-3′). The transmembrane and intracellular domains of human RANK was amplified with PCR using two primers (Forward, 5′-CTGGACCCCGATATCGGTTTAATAATTCTG CTTCTCTTCG-3′; Reverse, 5′-GAGGGGCGGA TCCTAAACAGCCTTGGCCCCGCCTT-3′) and pF1KE0079 (Kazusa DNA Res. Inst., Chiba, Japan), which is a plasmid containing the full-length RANK sequence, as a template. The linearized vector and amplified fragment were ligated using In-Fusion HD Cloning Kit (Clontech, Mountain View, CA), resulting in pL-SIN-EF1α-S-RANK-IP.

Cell culture A murine macrophage cell line RAW264 (RIKEN Cell Bank, Tsukuba, Japan; #RCB0535) was cultured in Minimum Essential Medium Eagle alpha modification (α-MEM) (Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% FBS (Life technologies, Carlsbad, CA, USA). A human embryonic kidney cell line 293T was cultured in Dulbecco's modified Eagle's Medium (DMEM) (Nissui Pharmaceutical Tokyo, Japan) supplemented with 10% FBS.

Gene transduction 293T cells were cultured in a 100 mm diameter dish overnight. 293T cells were co-transfected with pL-SIN-EF1α-HA-S-RANK-IP, pCMV-VSV-G, and pCMV-dR8.91 (gifts from Dr. Kenneth Rock, University of Massachusetts Medical School, Worcester, MA, USA) using Lipofectamine LTX (Life Technologies) according to the manufacturer's protocol. The culture medium was changed every day, and the viral supernatant was collected after 2 and 3 days of transfection. The collected supernatant (20 ml) was filtrated using 0.45 μm filters, and centrifuged at 6000 ×g for 16 h at 4°C. The lentiviral pellet was resuspended in 200 μl of serum-free DMEM. RAW264 cells were infected with the lentivirus in the presence of 10 μg/ml protamine sulfate (Wako Pure Chemical Industries, Osaka, Japan), followed by selection with 2 μg/ml puromycin (Sigma–Aldrich).

Surface expression of HA-tagged chimeric receptors Surface expression levels of HA-tagged chimeric receptors were measured by flow cytometry as described previously (37).

Differentiation Cells (2×10^3/well) were plated on a 48-well plate and cultured for 5 days with no ligand, 40 ng/ml RANKL (Wako Pure Chemical Industries), or 1 μg/ml BSA-FL (Sigma–Aldrich). The medium was replaced every 2 days.

TRAP staining Tartrate-resistant acid phosphatase (TRAP) staining was performed by using TRAP Staining Kit (Primary Cell Co., Sapporo, Japan) according to the instruction of the kit. The differentiated cells in the wells were washed with PBS and were fixed with 10% formalin for 2 min, followed by washing with MilliQ water three times. Five milliliters of tartaric acid-containing buffer (50 mM, pH 5.0) was added into the vial with the staining substrate. The staining substrate solution (200 μl) was added into each well and incubated at 37°C for 30 min. The cells were washed 3 times with MilliQ water. The cells were imaged with an Olympus CK2 inverted microscope and a Nikon Coolpix 995 digital camera. Multinucleated TRAP-positive cells were counted as osteoclasts.

Western blotting Cells (2×10^5) were plated in 6-well plates and cultured for two days. The medium was replaced with that containing 100 ng/ml RANKL (R&D systems, Minneapolis, MN) or 1 μg/ml BSA-FL. After 0, 2, 5, 7, or 10 min incubation, the cells were washed with ice-cold PBS. Lysate preparation and subsequent western blotting procedures were performed as described (20). Primary rabbit antibodies used are: anti-HA tag (Bethyl Laboratories, Montgomery, TX, USA), anti-JNK (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-IκBα (Cell Signaling Technology, Danvers, MA, USA), anti-p38 (Cell Signaling Technology), anti-MEK1/2 (Cell Signaling Technology) anti-β-tubulin (Santa Cruz Biotechnology), anti-phospho IκBα (Cell Signaling Technology), anti-phospho p38 (Cell Signaling Technology), anti-phospho JNK (Cell Signaling Technology), and anti-phospho MEK1/2 (Cell Signaling Technology).

RESULTS

Construction of S-RANK and establishment of RAW/S-RANK cells We have previously reported chimeric antibodies named signalobodies which induce growth, migration, or death signaling. On the basis of these studies, we constructed an S-RANK signalobody in which anti-FL scFv was fused with the extracellular D2 domain of EpoR and the transmembrane/intracellular domains of RANK (Fig. 1). The structure of RANK/RANKL co-crystals indicated that a RANKL trimer binds to the extracellular domain of RANK, which induces RANK trimerization and subsequent RANK activation. Therefore, an antigen needs to be at least trivalent and desirably multivalent to activate S-RANK efficiently. In our previous reports, we succeeded to activate an S-Fas signalobody through oligomerization with FL-conjugated BSA (BSA-FL) (25). This result indicates that FLs that are conjugated to BSA induced tri- or oligomerization of the signalobody, followed by signal induction. Therefore, we used BSA-FL, in which approximately 12 FL molecules were labeled per BSA molecule, as the antigen for activating S-RANK.

To demonstrate the concept of differentiation signalobody, we chose a murine macrophage cell line, RAW264, which could differentiate into osteoclast by the RANK activation. RAW264 cells were lentivirally transduced with the S-RANK expression vector, which encodes the HA-tagged S-RANK signalobody and IRES-puromycin^R cassette. S-RANK-expressing cells were selected with puromycin to establish RAW/S-RANK cells. To confirm the expression of S-RANK in RAW/S-RANK cells, western blot analysis was performed. As a result, the band for S-RANK was detected clearly (Fig. 2A). We also investigated the expression of S-RANK on the cell surface by staining the HA tag with mouse anti-HA antibody and