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A mitochondrial thioredoxin-sensitive mechanism regulates TGF-β-mediated gene expression associated with epithelial–mesenchymal transition

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ABSTRACT

Transforming growth factor (TGF)-β is a pro-oncogenic cytokine that induces the epithelial–mesenchymal transition (EMT), a crucial event in tumor progression. During TGF-β-mediated EMT in NMuMG mouse mammary epithelial cells, we observed sustained increases in reactive oxygen species (ROS) levels in the cytoplasm and mitochondria with a concomitant decrease in mitochondrial membrane potential and intracellular glutathione levels. In pseudo ρ0 cells, whose respiratory chain function was impaired, the increase in intracellular ROS levels was abrogated, suggesting an important role of mitochondrial activity as a trigger for TGF-β-stimulated ROS generation. In line with this, TGF-β-mediated expression of the EMT marker fibronectin was inhibited not only by chemicals that interfere with ROS signaling but also by exogenously expressed mitochondrial thioredoxin (TXN2) independent of Smad signaling. Of note, TGF-β-mediated induction of HMGA2, a central mediator of EMT and metastatic progression, was similarly impaired by TXN2 expression, revealing a novel mechanism involving a thiol oxidation reaction in mitochondria, which regulates TGF-β-mediated gene expression associated with EMT.

1. Introduction

Transforming growth factor (TGF)-β superfamily members including TGF-β isoforms, activins, inhibins, and bone morphogenetic proteins regulate various cellular functions ranging from embryonic development to adult tissue homeostasis, while under pathological conditions, they contribute to aggravation of diverse disorders such as tissue fibrosis and autoimmune diseases [1]. In particular, a wealth of studies has implicated TGF-β in tumorigenesis and characterized its ostensibly ambivalent roles during the process, depending on the stage of tumor development. According to a widely accepted view, in an early stage of carcinogenesis, TGF-β can suppress the neoplastic transformation of epithelial cells through growth inhibitory and proapoptotic activities. In advanced cancers, however, where the growth inhibitory signal of TGF-β is interrupted and/or distorted through genetic mutations in the molecular machineries that receive and/or transmit TGF-β signals, TGF-β stimulates neoplastic transformation through its ability to induce epithelial plasticity, thus leading to epithelial–mesenchymal transition (EMT) and/or cell motility [2,3]. Accordingly, TGF-β-mediated EMT induction has been a focus of cancer biology to better understand cancer progression.

TGF-β signals primarily through two pathways, Smad and non-Smad pathways [4]. Recently, additional pathways evoked by reactive oxygen species (ROS) have been associated with TGF-β signaling. Indeed, several reports demonstrated that TGF-β stimulates intracellular ROS production in various types of cells after the seminal discovery in murine osteoblastic cells [5,6]. According to the reports, ROS-evoked signals play a role in TGF-β-mediated EMT in breast and renal tubular epithelial cells and cardiac and pulmonary fibrosis [7–10] dependently or independently of the Smad pathway, although molecular details remain to be elucidated in most cases.

In an effort to substantiate the biological significance of ROS signals in TGF-β biology, we studied an increase in ROS levels and the impact of a consequent change in cellular redox on TGF-β-mediated gene expression associated with EMT. A series of analyses with mammary epithelial cells undergoing EMT after TGF-β treatment revealed a sustained increase in ROS levels accompanying reduction of cellular redox in cells, highlighting mitochondria as important players. Of interest, a cysteine thiol-disulfide exchange reaction in mitochondria is suggested to be involved in TGF-β-mediated regulation of gene expression. As a prominent example,

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induction of HMGA2, a high mobility group AT-hook 2, which mediates TGF-β-induced EMT, was suppressed by the exogenous expression of TXN2, a mitochondrial thioredoxin. In summary, mitochondria-directed cellular redox changes have emerged as important regulators in TGF-β-mediated gene expression associated with EMT.

2. Materials and methods

2.1. Cell culture

NMuMG mouse mammary epithelial cells were obtained from ATCC and cultured as described previously [11]. The cells were treated with TGF-β1 (R&D Systems, Minneapolis, MN, USA) in conditioned media. Chemicals [12] were pretreated for 30 min before TGF-β1 treatment. Establishment of pseudo ρ0 cells was described previously [13].

2.2. Expression plasmids

HyPer-C and -M retrovirus plasmids were constructed by inserting cDNA from pHyPer-cyto and -dMito (Evrogen, Moscow, Russia) [14] into pMXs-IN [15]. Lentiviral expression vectors, CSII-CMV-MCS-IRES2-Bsd and hTERT/pLXIN, were kindly gifted by Dr. H. Miyoshi (Riken BRC, Japan) and Dr. H. Tahara (Hiroshima University, Japan), respectively. The Tet-Off lentiviral expression vector, CSII-TREII, was constructed by ligating a tetracycline-inducible unit from pSIN-TREII and tTA advanced/pMXs-IP to CSII-CMV-MCS-IRES2-Bsd [16]. Human thioredoxin (TXN)2 cDNAs were amplified from the human mammary epithelial cell cDNA library and inserted into the CSII-TREII vector.

2.3. Infection

Retrovirus production and infection was described previously [16]. For the generation of lentivirus-expressing HEK293T cells (Riken BRC Cell Bank, Tsukuba, Japan), the CSII-TREII vector was cotransfected with packaging plasmids (pCAG-HIVgp and pCMV-VSV-G-RSV-Rev) [17] by the calcium phosphate precipitation method. The conditioned medium containing viral particles was harvested 48 h after transfection and used to infect cells with 4 μg/ml polybrene and 10 ng/ml doxycycline (Dox). Successfully infected cells were selected and maintained with 5 μg/ml puromycin in the presence of 10 ng/ml Dox.

2.4. Immunoblotting

The immunoblotting procedure was described previously [11]. The primary antibodies used are listed in Supplementary data.

2.5. Luciferase reporter assay

Cells in 12-well plates were transiently transfected with 0.5 μg of luciferase constructs, together with 0.02 μg of phRL/CMV (Promega, Madison, WI, USA), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Luciferase activities were measured using the Dual Luciferase Assay Kit (Promega), according to the manufacturer's instructions. SBE/Luc ($(CAGA)_{12}$ MLP Luc) was a generous gift from Dr. J.-M. Gauthier [18]. pAP1/Luc was purchased from Stratagene (La Jolla, CA, USA).

2.6. RNA extraction and real-time reverse transcription PCR

RNA extraction and real-time reverse transcription (RT) PCR were performed in the same manner as described previously [16]. Quantities of mRNA were normalized by mRNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers used are described in Supplementary data.

2.7. Measurement of intracellular redox states and mitochondrial membrane potential

Measurement of the intracellular redox state with H_2DCF was described previously [19]. For the measurement of the subcellular redox state, cells were infected with the HyPer-C or -M retrovirus and after 24–48 h, emission at 490 nm was observed in a single cell at intervals of 4 h using a Nikon ECLIPSE TE2000-U microscope equipped with a high-speed cooled digital CCD camera (Nikon Corporation, Tokyo, Japan). Images were acquired using an AquaCosmos image acquisition and analysis system (Hamamatsu Photonics, KK Hamamatsu, Japan). Mitochondrial membrane potential ($\Delta\psi m$) and intracellular glutathione (GSH) levels were determined using the Mito-ID Membrane Potential Cytotoxicity Kit (Enzo Life Science, Farmingdale, NY, USA) and GSH-Glo Glutathione Assay Kit (Promega), according to the manufacturer's instructions, respectively.

3. Results

3.1. Increase in ROS levels in the cytoplasm and mitochondria of mammary epithelial cells treated with TGF-β

We examined the changes in intracellular ROS levels in TGF-β-treated NMuMG cells using two distinct types of fluorescent probes sensitive to cellular redox. One is H_2DCF, a chemical dye routinely used for ROS detection in cells, and the other is HyPer-C, a genetically encoded probe that is expressed as a compound protein consisting of a derivative of green fluorescent protein and a regulatory domain of a bacterial ROS sensor OxyR, directed to localize in the cytoplasm [14]. The results obtained from each probe are shown in Fig. 1A and B, respectively, indicating that the fluorescence intensities of both probes changed essentially in the same patterns. In normal cells, they underwent a sustained increase followed by a decline at 24 h, indicating that the cytoplasmic ROS levels were increased over 20 h after TGF-β treatment (Fig. 1A and B, Normal). The increase was biphasic with peaks observed at approximately 8 and over 16–20 h, implying that multiple ROS-generating systems were involved in the elevation under the conditions [20].

Among numerous potential sources, mitochondria are regarded as a major site of ROS production within cells. Thus, we tested their involvement in the above change using pseudo ρ0 cells in which the respiratory chain function is severely deteriorated [19]. In contrast to ROS levels in the normal cells, changes in ROS levels in the ρ0 cells appeared within a margin of error (Fig. 1A and B, ρ0). The absence of the response to TGF-β in the ρ0 cells was unlikely to be due to a fundamental loss of responsiveness to TGF-β because phosphorylation of Smad3 and the subsequent signal transduction were intact in the cells (Supplementary Fig. S1A and B). Taken together, mitochondrial activity was indispensable for the increase in the cytoplasmic ROS levels in the normal cells, and mitochondria were prospective sources and/or triggers of the increased cytoplasmic ROS levels.

Therefore, we next examined changes in mitochondrial ROS levels after TGF-β treatment using another genetically encoded fluorescent probe, HyPer-M [21], a mitochondria-targeted version of HyPer-C. TGF-β treatment caused an increase in fluorescence over several hours (Fig. 1C), suggesting that the mitochondrial ROS levels also increased under the conditions. However, different from the changes in HyPer-C (Fig. 1B), the intensity was elevated with

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