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Intracellular Redox and Mitochondria Regulation by Transforming Growth Factor-β-Its Implication in Induction of Epithelial-Mesenchymal Transition

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Introduction

Transforming growth factor-β (TGF-β) is a cytokine that is profoundly related to tumorigenesis. We found that TGF-β stimulation increases the intracellular levels of reactive oxygen species (ROS) and alters the intracellular redox potential. In mammary gland epithelial cells treated with TGF-β, a mitochondria-dependent increase in the ROS concentration occurs in a sustained manner, while the mitochondrial membrane potential and intracellular glutathione (GSH) level decrease. To gain insights into the significance and mechanism of this response to TGF-β, we determined that approximately 15% of TGF-β-inducible genes are affected by mitochondrial depletion and therefore, we conducted further detailed analyses. Interestingly, we found that TGF-β-mediated expression of the epithelial–mesenchymal transition (EMT) marker fibronectin is inhibited by exogenously expressed mitochondrial thioredoxin (TXN2) independent of Smad signaling. Of note, the induction of high mobility group AT-hook 2 (HMGA2), a central mediator of EMT and metastatic progression, is similarly inhibited by TXN2 expression, revealing a novel mechanism involving a thiol oxidation reaction in mitochondria that regulates TGF-β-mediated gene expression associated with EMT.

Mitochondria-dependent production of reactive oxygen species and intracellular redox changes following stimulation with transforming growth factor-β.

Transforming growth factor-β (TGF-β) signals, which are primarily classified into Smad and non-Smad signals, have been found to be increasingly complex, reflecting the diversity of biological activities of TGF-β. In the late 1980s, we found that reactive oxygen species (ROS) are produced at increased levels in cells stimulated with tumor promoters [1] and subsequently, in murine osteoblasts stimulated with TGF-β [2]. Over the last few years, we studied the relationship between TGF-β-mediated induction of malignant transformation and ROS production [3]. For this purpose, we first reinvestigated ROS production in murine mammary gland epithelial cells (NMuMG) using HyPer, an ROS-sensitive fluorescent protein [4], in addition to the fluorescent probe 2′,7′-dichlorodihydrofluorescein (H$_2$DCF). Cytoplasmic and mitochondrial forms of HyPer were intracellularly expressed; thus, we determined their fluorescence intensities over time and found that treatment with TGF-β1 continuously increased the fluorescence intensity of the cytoplasmic form, which was in agreement with the observations using H$_2$DCF. Thus, TGF-β1 induced a sustained elevation of ROS production in epithelial cells (equivalent to high micromolar concentrations of H$_2$O$_2$) from approximately 1-2 to 24 h after stimulation, which differed from the transient increase previously observed in mesenchymal cells within 2 h after stimulation [2]. Although transient, fluorescence of the mitochondria-localized

HyPer also increased and peaked at approximately 12 h. These changes were mediated by the TGF-β receptor, as shown, using a system to ectopically express a dominant-negative form of the type I receptor ALK5. Interestingly, in cells that underwent inhibition of mitochondrial DNA replication/transcription to decrease mitochondrial activities, the increase in cytoplasmic ROS levels was no longer observed, suggesting that cytoplasmic change in ROS levels require mitochondrial activities.

In conjunction with the observation that ROS levels in mitochondria increased, the aforementioned results suggested that the source of ROS production following TGF-β stimulation was likely the mitochondria in mammary epithelial cells. In 2005, Yoon et al. demonstrated that TGF-β induces prolonged mitochondrial ROS generation through decreased complex IV activity [5]. We studied the possible contributions of other systems (such as NADPH oxidase) using small molecule inhibitors but were unable to draw firm conclusions because of insufficient specificity of inhibitors. On the other hand, study of the mitochondrial membrane potential (ΔΨm) indicated that ΔΨm decreased by 20%–50% from 8 to 12 h and continued to decrease until 48 h after TGF-β stimulation [3]. Among respiratory chain complexes, the activity of Complex I significantly decreased. These observations suggested that TGF-β affected mitochondrial functions accompanying the increased ROS production, although precise mechanisms remain unknown. No changes in levels of mitochondrial proteins were observed, suggesting the absence of significant changes in the amount or structure of mitochondria. In addition, we investigated the expression of Mn superoxide dismutase (MnSOD), a mitochondrial protein that is induced in response to oxidative stress, and detected no changes. This result suggested that the TGF-β-stimulated increase in ROS levels was below the level that induces oxidative stress. In addition to NMuMG, we found that these changes occurred in normal human mammary epithelial cells and breast cancer cell lines. Other researchers also found that TGF-β promoted mitochondrial ROS production in alveolar epithelial cells [6], bone marrow mesenchymal stem cells [7], and podocytes [8].

In parallel with the increase in ROS levels, the concentration of glutathione (GSH), a representative cellular redox buffer, decreased from 20 h after TGF-β stimulation. Moreover, DNA microarray analysis indicated that expression levels of approximately 30 genes involved in GSH metabolism were markedly reduced when cells were treated with TGF-β for 24 h. Therefore, the intracellular redox environment of TGF-β-stimulated cells is considered to be shifted toward the oxidative state, possibly because of a combined effect of enhanced mitochondrial ROS production and control of GSH metabolism at the gene expression level. Similarly, other studies have suggested that TGF-β shifts the intracellular redox potential toward the oxidative state. For example, TGF-β increases ROS production in

hepatocytes through NADPH oxidase and mitochondrial respiratory chain complex I and decreases the levels of antioxidative enzymes, such as catalase and MnSOD [9,10]. In a TGF-β-stimulated alveolar epithelial cell line (A549), intracellular ROS levels increased as the GSH concentration decreased, and expression levels and activity of the rate-limiting enzyme in the pathway of GSH synthesis (γ-glutamyl–cysteine synthetase γ-GCS) decreased [11].

Toward identification of the mechanism and significance of Mitochondrial/ROS signalling.

The changes in cellular redox potential toward an oxidative state may simply represent an early stage of apoptosis. However, this is unlikely considering that during the induction of apoptosis by ROS, outer membrane permeability is enhanced [12], and the change that we observed was related to the permeability (membrane potential) of the inner membrane ($\Delta\Psi m$), at least within the time interval studied. Furthermore, decreased inner membrane potential is not usually required for the induction of apoptosis because it occurs only at a late stage of apoptosis. In addition, in our system, increased cell death was barely detectable at 24 h after TGF-β stimulation, and the activation of apoptotic pathways, such as caspase-9, was only detectable after 48 h [13]. Therefore, assuming that the changes that occurred before 24 h were not the initial changes in apoptosis, but rather served as signals; we named these the Mitochondrial/ROS pathway. Presumably, the Mitochondrial/ROS pathway may play a role in the induction of morphological changes, such as epithelial–mesenchymal transition (EMT), or regulation of gene expression before determination of cell fate according to the context of cells while balancing proapoptotic and antiapoptotic activities. In lung fibroblasts, TGF-β-induced mitochondrial ROS production was implicated in fibrosis [6,14] and induction of senescence in other cases[5,7]. Involvement in mitophagy regulation was also reported [6].

To evaluate the significance of the Mitochondrial/ROS pathway in TGF-β signaling and understand the physiological role of the pathway in more detail, we analyzed changes in gene expression profiles induced by TGF-β in mitochondria-deficient or mitochondrial DNA-less, so called pseudo-ρ0, cells (unpublished data). Even in the mitochondria-deficient cells, TGF-β signaling, which is mediated by Smad3 phosphorylation and p38MAP kinase activation, and intranuclear transcriptional activity (monitored using the respective reporters for SBE, 3TP-Lux, and PAI-1) appeared normal under TGF-β stimulation. Such pseudo-ρ0 cells were stimulated with TGF-β to comprehensively investigate changes in gene expression profiles, which showed that expression of certain genes was significantly inhibited (e.g., MMP9) or enhanced (e.g., PAI-1). In summary, the results showed that the levels of approximately 15% of TGF-β-responsive genes were markedly affected by over two-fold, depending on levels of mitochondrial activities, suggesting the significance of the Mitochondrial/ROS pathway in the regulation of gene expression by TGF-β.

However, the above experimental system was based on a considerable decrease in mitochondrial respiratory activities, which may produce stress signals that modify TGF-β signaling. Therefore, the significance of the Mitochondrial/ROS pathway should be investigated using a more sophisticated system. Accordingly, we developed a molecular genetic system to specifically regulate intracellular and mitochondrial redox potential [3]. Using such tools as expression systems for cytoplasmic and mitochondrial-targeted CuZnSOD, MnSOD, and catalase, we detected no significant effects on TGF-β-mediated gene expression or EMT. Instead, we recently found that

TGF-β-induced morphological changes (EMT) tend to be suppressed when cells are manipulated to express mitochondria-localized thioredoxin (TXN2) [3]. In line with this finding, expression of the EMT marker fibronectin was inhibited by TXN2 expression. Of note, the induction of HMGA2 (high mobility group AT-hook 2), a central mediator of EMT and metastatic progression, was similarly inhibited by TXN2 expression, revealing a novel mechanism involving a thiol oxidation reaction in mitochondria that regulates TGF-β-mediated gene expression associated with EMT (Figure 1).
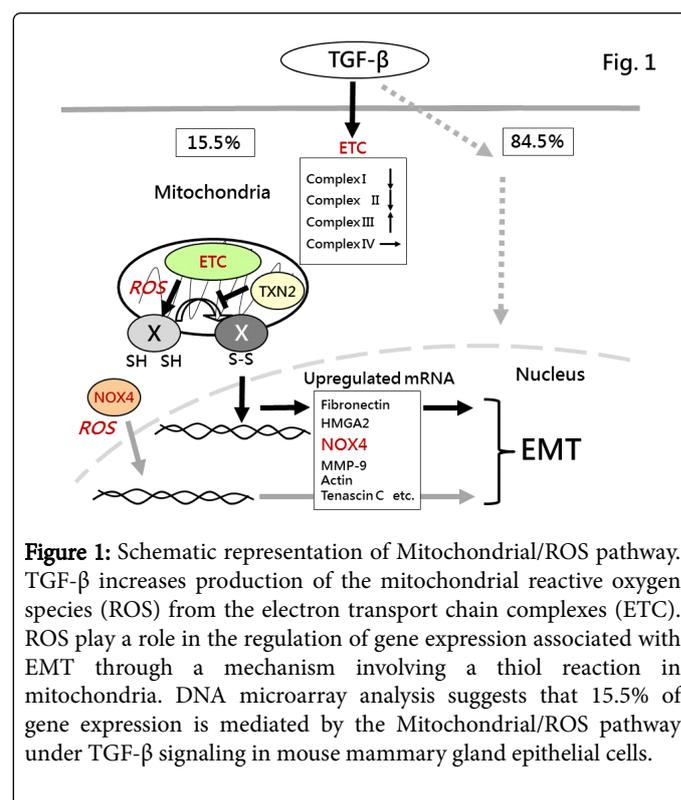

Figure 1: Schematic representation of Mitochondrial/ROS pathway. TGF-β increases production of the mitochondrial reactive oxygen species (ROS) from the electron transport chain complexes (ETC). ROS play a role in the regulation of gene expression associated with EMT through a mechanism involving a thiol reaction in mitochondria. DNA microarray analysis suggests that 15.5% of gene expression is mediated by the Mitochondrial/ROS pathway under TGF-β signaling in mouse mammary gland epithelial cells.

Thus, the importance of ROS signaling has emerged in regulation of malignant transformation of cells by TGF-β. Several recent studies also suggested a role of ROS signaling in TGF-β-induced EMT [15-17]. These studies characterized the NADPH oxidase 4 (NOX4) as a source of ROS under TGF-β stimulation, which is apparently different from our findings. However, mitochondrial ROS possibly mediates NOX4 induction by TGF-β [14], implying that the TGF-β-stimulated NOX4 expression observed in the above-mentioned studies may be caused by mitochondrial ROS production prior to expression. Consistent with these findings, we observed that NOX4 was induced by TGF-β1 in a redox-sensitive and mitochondria-dependent manner in NMuMG cells (unpublished data), suggesting that ROS are eventually produced by the two generating systems and collaborate to induce cellular changes. Further studies are needed to clarify the relationship between mitochondrial ROS and NOX4 expression in more detail, and the precise roles of ROS produced by each system in regulation of EMT.

Conclusion

A line of evidence indicates an imbalance in the intracellular redox potential in several types of cancer cells, leading to increased attention to the relationship between mitochondrial activity and malignant transformation. Mitochondrial/ROS signaling in TGF-β-induced EMT,

therefore, is of great interest in cancer biology research. Given the possible involvement of mitochondrial ROS in NOX4 regulation, the signaling pathway is assumed to be made up of mitochondrial respiratory complexes, NOX4, and ROS, which would be termed Mitochondrial/NOX4/ROS signaling accordingly. We expect that further elucidation of the Mitochondrial/ (NOX4)/ROS pathway will contribute to better understanding of cancer cell phenotypes controlled by TGF-β.

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