Analysis of oxidative stress response genes in HUC-F2

Yuri Shimizu

Oxygen is both essential for aerobic life, being the terminal electron acceptor in respiration, and hazardous, because of the potency of reactive oxygen species (ROS). ROS include the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and singlet oxygen, which are produced by neutrophils and macrophages in the human body. At excess concentrations or in the wrong location, ROS can damage proteins, carbohydrates, polyunsaturated fatty acids and DNA, and lead to a variety of degenerative processes and diseases. Apoptosis, a programmed cell death process characterized by distinct morphological and biochemical changes that take place upon the activation of a family of serine proteases known as caspases plays important roles in the development, homeostasis and anticancer defence of multicellular organisms. ROS have been shown to induce apoptosis in several different cell systems. In this paper, we examined what enzymes are involved in defense system against oxidative stress produced by H_2O_2 using new type of micro array, and confirmed by real time PCR method. Studies of gene expression may help to determine the network of genes involved in the oxidative stress defense system.

First, the effect of H_2O_2 on expression of genes in normal umbilical card fibroblast cells was investigated by treating HUC-F2 cells with 100μM H_2O_2 for 4 h and analyzing induction of mRNAs using Agilemt whole human genome array. This experiment was repeated three times. The expression of nuclear receptor subfamily 4, group A, member 1 (NR4A1), a member of an orphan nuclear receptor superfamily, was induced >2 fold. Real-time PCR was used to confirm induction of NR4A1 genes in HUC-F2 cells and the mRNA expression of NR4A1 was increased by H_2O_2 concentration dependently.

NR4A1 is widely expressed but primarily detected in the thymus, osteoblast, liver, and pituitary gland. NR4A1 was originally recognized for its role in the regulation of cell survival and differentiation. However, Expression of NR4A1 is induced by multiple stimuli in different cell types, and NR4A1 has been shown to trigger

apoptosis in a number of cell lineages exposed to proapoptotic stimuli by directly targeting mitochondria and inducing cytochrome c release. Paradoxically, NR4A1 is also reported to act as a death inhibitory factor, blocking cell death induced by ceramide or tumour necrosis factor (TNF). The finding to date support important roles of NR4A1 in cell transformation and tumourigenicity via both their anti-apoptotic and pro-apoptotic functions. Although apoptotic and carcinogenic roles of NR4A1 have been discussed with respect to its potential as a therapeutic target in cancer cells, limited reports of NR4A1 activity in normal human cell of HUC-F2 are documented. Therefore, in this study I focused on the mechanisms that determine whether NR4A1 exerts pro- or anti-apoptotic activity in normal human cells.

The involvement of NR4A1 in cell death induced by oxidative stress was determined by transient transfection of HUC-F2 cells with NR4A1-specific siRNA. NR4A1 expression was significantly reduced upon transfection of cells with NR4A1-targeting siRNA, compared with control siRNA at 48 h when the siRNA sequence GGUCCCUGCACAGCUUGCUUGUCGA' was used. Si-NR4A1-transfected HUC-F2 cells were subsequently challenged with 0-300 μM H_2O_2 for 24 h. Growth of HUC-F2 cells transfected with si-NR4A1 was significantly reduced following exposure to 300 μM H_2O_2 for 24 h, as measured with the MTT assay.To compare the proportion of early apoptotic cells, HUC-F2 cells were transfected with siRNA and treated with 200 μM H_2O_2 at concentrations below that leading to reduced cell viability. Apoptosis was determined using flow cytometry (FCM). HUC-F2 cells were transfected with si-NR4A1 for 48 h, treated with 200 μM H_2O_2 for 2 h and measured with FCM. The peak of the annexin V‑FITC intensity histogram for cells treated with si-NR4A1 and H_2O_2 was shifted rightward, compared with the control and the percentages of early apoptotic cells transfected with si-NR4A1 and treated with H_2O_2 to 9.0% from 7.3% (si-NC with H_2O_2, P=0.013). This result suggested a prosurvival or anti-apoptotic function of NR4A1.

Caspase activation plays a central role in the execution of apoptosis. The key components of the biochemical pathways of caspase activation have been identified. Two pathways of caspase activation have been

extensively characterized to date, specifically, cell surface death receptor and the mitochondria-initiated pathways. In the cell surface death receptor pathway, activation of caspase-8 following its recruitment to the death-inducing signalling complex is the critical event that transmits the death signal. In the mitochondria-initiated pathway, caspase activation is triggered by the formation of a multimeric Apaf-1/cytochrome c complex that is fully functional in recruiting and activating procaspase-9. Activated caspase-8 and -9 cleave and activate downstream caspases-3. In this study, activities of caspase-3 and -8 of NR4A1 knockdown cells treated with 200 μM H_2O_2 were significantly higher than those of negative control cells. Caspase-9 activity was also elevated, but not to a significant extent. This data suggest that NR4A1 inhibits extrinsic apoptosis by reducing caspase-8 and caspase-3 activities.

In this study, we showed that NR4A1 acts as a regulator that inhibits extrinsic apoptosis in HUC-F2 cells during oxidative stress by reducing caspase-8 and -3 activities.