Respiratory burst of granulocytes

While it is well known that polymorphonuclear leukocytes play essential role in host defence against microorganisms, mechanisms by which granulocytes may be involved in the immune response against cancer are not well understood. Recently, it was reported that spontaneous regression/complete resistance to cancer cells is mediated by rapid infiltration of leukocytes, mostly as a consequence of innate immune response. In addition, the administration of granulocytes at the site of solid tumors can lead to tumor regression or can slow down tumor growth and extend the overall survival of animals. The activation process of granulocytes is accompanied by the intense production of reactive oxygen species (ROS) resulting in oxidative stress. ROS are normally formed in small quantities during metabolic processes. The level of cellular ROS that induces initial effects leading to protection of cells is only slightly lower than the levels that cause awful effects. Overproduction and accumulation of ROS is cytotoxic and damages macromolecules (DNA, proteins, sugars and lipids), causing degeneration of tissues, premature aging, apoptosis, cellular transformation, mutagenicity, and cancer. Both prokaryotic and eukaryotic cells respond to this toxicity...
by the protection mechanisms coordinately inducing a series of genes encoding detoxifying and antioxidative stress enzymes/proteins that provide the necessary protection against oxidative and electrophilic stress12-15. In eukaryotes, there are transcription factors known to be activated by ROS. Those transcription factors include NF-E2-related factors (Nrfs)12. This review focuses on Nrf2 that regulates coordinated activation of a variety of genes in response to oxidative stress.

Nuclear factor-erythroid 2 (NF-E2)

NF-E2 belongs to the bZip (basic region leucine zipper) transcription factors. NF-E2 was purified from mouse erythroleukemia (MEL) cells and is composed of 45 kDa and 18 kDa subunit16. NF-E2 is expressed only in erythroid cells, megakaryocytes, and mast cells. It binds to NF-E2 recognition site (GCTGAGTCA) and regulates tissue specific expression of the globin genes15,16. NF-E2 functions as a heterodimer with small Maf proteins that are ubiquitously expressed17. Maf proteins are a family of nuclear transcription factors. Maf family constitutes from several genes (v-maf, c-maf and other v-maf related genes) and can be divided into large and small Maf subfamilies. They can act both as activators and repressors of a number of eukaryotic genes18-20.

NF-E2 related factors (Nrfs)

Screening for a molecule that can interact with the NF-E2 binding motif led to the identification of NF-E2 related factors (Nrf1, Nrf2, and Nrf3)21. All of these factors have highly conserved cap’n’colar (CNC) region, bZip region, basic and acidic region21,22. It is known that the basic region is responsible for DNA binding, and that the acidic region is required for transcriptional activation. Many researchers have demonstrated Nrf2 to be the most prominent factor in activation and induction of antioxidant responsive element (ARE)- mediated genes in comparison with Nrf1 and Nrf323,24.

Nrf2, a 68 kDa protein21, consists of six highly conserved domains, Neh1-6. Few years ago Li et al characterized in Nrf2 a nuclear export signal (NES) in the leucine zipper domain (NESzip)25 and in the Neh5 transactivation domain (NES\textsubscript{TA}26. A nuclear localization signal (NLS) was also identified as a bipartite NLS in the basic region (bNLS) of Nrf225,27, or a monopartite at the N-terminus (NLS\textsubscript{N}) and C-terminus (NLS\textsubscript{C}) of Nrf228. Based on the function of multiple Nrf2 NES/NLS motifs in the non-stimulated and oxidative stress conditions, Li et al proposed a new Keap1-independent Nrf2 signaling model26,29. In this model, Nrf2 is a redox sensitive and consists of a constitutively active NESzip bNLS-NLS\textsubscript{N}-NLS\textsubscript{C} tandem and a conditional NES\textsubscript{TA} motif28. Oxidative stress signals influence the reactive Cys in the NES\textsubscript{TA} and disable the NES\textsubscript{TA} resulting in Nrf2 nuclear translocation29.

The Keap1-dependant Nrf2 signaling will be discussed later.

Detoxifying enzymes and antioxidant responsive element

Against damage from ROS and xenobiotics, cells and tissues defend by enhancement of detoxifying enzymes. The generation of superoxides and electrophiles is a result of antioxidants and xenobiotics metabolism. There are two major groups of metabolizing enzymes, phase I (activation) and phase II (detoxifying) metabolizing enzymes. Phase I enzymes include oxidation, reduction, and hydrolysis. Throughout phase I detoxication structurally diversified chemicals are, with cytochrome P450 mono-oxygenase system, oxidated into their metabolically activated forms. Phase II include glucuronidation, sulfation, acetylation, methylation and conjugation with glutathione. Phase II detoxicating enzymes are induced by phase I metabolites as well as by antioxidants, which are very often electrophilic. Throughout phase II detoxification, the activated substrates are catalysed into nontoxic metabolites30.

Phase II enzyme genes are induced through the ARE31. This important regulatory element was also called the electrophile responsive element (EpRE)32. The ARE was identified in the regulatory region of some genes33-36, and was also recognized as a DNA element that regulates basal expression and coordinated induction of genes encoding antioxidant enzymes in response to antioxidants and xenobiotics12. The ARE sequence plays an essential role in the regulation of the cellular defense system. The minimum ARE core sequence was reported to be 5'-TGACNNNGC-3'. Other neighboring sequences and elements also affect ARE-mediated expression and induction of antioxidant genes37,39.

Nrf2 plays an important role in the resistance to xenobiotic toxicity by the regulation of ARE-mediated gene expression40,41. In response to antioxidants, xenobiotics, metals, and UV irradiation, Nrf2 protein binds strongly to ARE sequence and regulates ARE-mediated antioxidant enzyme gene expression and induction13,42,43.
In the interaction between Nrf2 and ARE sequence, Nrf2 requires other cofactors, by forming heterodimer with a member of the small Maf proteins (MafK and MafG)\textsuperscript{41,44}. Dimerization with one of the small Maf proteins (transactivation) allows Nrf2 binding to the ARE. Some studies have shown the role of Nrf2-MafK heterodimers in the activation of ARE-mediated gene expression\textsuperscript{41}.

**Keap1 acts as a negative regulator of Nrf2 (Keap1-Nrf2 complex)**

Keap1 (Kelch-like ECH-associated protein 1) or INrf2 (inhibitor of Nrf2) is a cytosolic inhibitor of Nrf2\textsuperscript{45,46}. Keap1 is a dimer and retains Nrf2 in the cytoplasm. Keap1 was identified as a direct binding partner of Nrf2, using the Neh2 domain as a bait\textsuperscript{45}. N-terminal Neh2 domain is necessary for the recruitment of Nrf2 negative regulator (Keap1). Keap1 consists of five domains. One of them is Kelch domain or the double glycine repeat (DGR) domain, through which Keap1 binds Neh2 and actin\textsuperscript{45,47}. Normally, Nrf2 associates with Keap1 in the cytoplasm and represses Nrf2 transactivation activity\textsuperscript{45}. After direct attack by electrophiles or ROS or indirect actions such as phosphorylation, Nrf2 is released from Keap1 repression, translocated into nucleus where it activates the transcription of a variety of detoxifying enzyme genes through the ARE\textsuperscript{45}. After dissociation from Nrf2, Keap1 remains in the cytosol\textsuperscript{46}. Under basal conditions, Nrf2 is present at low concentrations due to CUL3-dependent E3-ubiquitin ligase mediated ubiquitination of Nrf2\textsuperscript{48}. Mutational studies revealed three Keap1 cysteine residues (Cys 151, Cys 273 and Cys 288)\textsuperscript{49} that are essential for degrading Nrf2 by Keap1-mediated ubiquitination. Nrf2 dissociation and Keap1 ubiquitination, mediated by electrophiles or ROS, are abolished by the mutation of Cys 151\textsuperscript{49}. This finding was further verified in vivo\textsuperscript{50}. The mechanisms by which Cys 273 and Cys 288 affect Keap1 function are not well understood\textsuperscript{51}. Recently, Chen et al\textsuperscript{52} described that interaction between Nrf2 and p21 interferes with Keap1-Nrf2 binding and thus upregulates Nrf2-mediated antioxidant response. The antioxidant function of p21, mediated through activation of Nrf2 by stabilizing the Nrf2 protein, was further verified by in vivo studies using p21-deficient mice\textsuperscript{52}.

Several reports have shown that Nrf2 binding to Keap1 leads to degradation of Nrf2\textsuperscript{53-56}. After Nrf2 is released from Keap1, it has a strong transactivation potential such as before binding with Keap1, which suggests that any modification of Nrf2 is only required for the release of Nrf2 from Keap1\textsuperscript{56}. Under oxidative stress conditions, besides Keap1-dependent degradation, Nrf2 can be also degraded by proteosomal degradation (Keap1-independent degradation)\textsuperscript{55,57}.

There is an autoregulatory loop, Nrf2:INrf2 (Keap1), between stress sensors Keap1 and Nrf2 in the Nrf2 pathway\textsuperscript{58}. Namely, ARE that binds to Nrf2 regulates Keap1 gene expression and induction and Keap1 controls Nrf2 by serving as an adaptor for degradation\textsuperscript{59}.

**The involvement of MAPKs, PKC, PI3K and GSK-3β in the activation of Nrf2**

The mitogen-activated protein kinases (MAPK) are important cellular signaling components that transform various extracellular signals into intracellular responses through phosphorylation cascades. MAPK pathways that are activated by MEKK1, TAK1, and ASK1 may link chemical signals to Nrf2 which then leads to the activation of ARE-dependent genes\textsuperscript{60}. The correlation between MAPK activation and Nrf2-mediated detoxifying enzyme suggests that Nrf2 could be a downstream target of activated MAPKs. If MAPK are inhibited prior to exposure to detoxifying enzyme inducers, Nrf2 accumulation is decreased\textsuperscript{64} and Nrf2 translocation to the nucleus is reduced\textsuperscript{61}. Recently, it was reported that chemosensitivity of lung cancer cells to anti-cancer agents may be modulated by HO-1 through the MAPK-Nrf2 pathway\textsuperscript{62}.

The importance of protein kinase C (PKC) is in phosphorylation of many target proteins that control cell growth and differentiation. A classic PKC activator can activate ARE-mediated gene expression and this activation can be inhibited by PKC inhibitors\textsuperscript{63}. Posttranslational modification of Nrf2 may also represent an important regulatory step in the activation of Nrf2, for instance, phosphorylation of Nrf2\textsuperscript{63}. PKC phosphorylation site in Nrf2 protein is Ser-40\textsuperscript{64} and is located in the Keap1-interacting Neh2 domain. The phosphorylation of this site by PKC disrupts the interaction of Nrf2 with Keap1 leading to Nrf2 release from Keap1-Nrf2 complex. This phosphorylation is not necessary for Nrf2 stabilization or transcriptional activation of ARE-mediated gene expression\textsuperscript{56,64}.

Phosphatidylinositol 3-kinase (PI3K) is important in cell growth, differentiation and apoptosis. PI3K is involved in the regulation of ARE and detoxifying enzymes: when PI3K is inhibited, ARE reporter expression is also inhibited. Overexpression of constitutively active PI3K always leads to the activation
of ARE activity in a dose dependent manner. This up-regulatory effect was completely blocked by dominant-negative Nrf2. A partial explanation of PI3K function in the cytoprotection machinery is that PI3K is involved in the regulation of nuclear translocation of Nrf2 protein. PI3K regulates Nrf2 through actin rearrangement in response to oxidative stress.

A delayed response of electrophilic/oxidative stress activates glycogen synthase kinase-3 beta (GSK-3β) that acts upstream of Fyn kinase controlling the nuclear export of Nrf2. Phosphorilation of Fyn, mediated by GSK-3β, is followed by phosphorilated Fyn translocation into the nucleus where it exerts its activity by phosphorilating Nrf2 at Tyr 568 thus leading to nuclear export and degradation of Nrf2. GSK-3β was shown to be essential in the down-regulation of the antioxidant cell defense elicited by Nrf2 after oxidant injury.

**Nrf2 in cancer prevention/promotion**

Many studies have described the role of Nrf2 in cancer prevention. Numerous chemopreventive compounds have been identified as Nrf2 inducers (e.g., sulforaphane) and their list is continuously growing. After their application, Nrf2-dependant adaptive response is induced, thus exerting its protective role from genotoxic damage caused by carcinogens. The role of Nrf2 in cancer prevention was confirmed by in vitro studies on Nrf2-null mice that had enhanced sensitivity to carcinogens in comparison to wild-type mice. The incidence and multiplicity of tumors as well as the tumor volume were enhanced in Nrf2-null mice. However, in the last few years there is a growing papers describing the tumor promoting role of Nrf2 proposing its dual role in cancer. Nrf2 overexpression was found in the later stages of cancer.

Furthermore, Nrf2 was found to be upregulated in hepatocellular carcinoma. Somatic mutations of Keap1 and low level expressions of Keap1 were identified in lung cancer tissues and cell lines, leading to enhanced nuclear accumulation and constitutive activation of Nrf2. These results suggest that elevated Nrf2 could play a role in the evolution of cancer. Moreover, Shibata et al found Keap1 gene alterations in biliary tract and gallbladder cancer. These alterations were especially frequent in gallbladder cancer leading to a loss of Nrf2 repression activity and constitutive activation of Nrf2. Finally, Keap1 mutation that impairs its ability for Nrf2 repression was also found in breast cancer. However, another recently published study revealed that most of breast cancer specimens examined had high Cul3/lower Nrf2 signature that can significantly contribute to increased cellular sensitivity to chemotherapeutic drugs. Nevertheless, to date, there is no evidence suggesting that the compounds used to induce Nrf2 signaling pathway have adverse impacts on tumor growth.

The involvement of oxidative stress in Nrf2 signaling pathway in an early stage of cancer is discussed below.

**The involvement of granulocyte respiratory burst in Nrf2 pathway in cancer**

ROS are reported to have an important role in tumor biology. Persistent oxidative stress may activate antioxidant systems, constitutively activate transcription factors and induce expression of proto-oncogenes. Furthermore, it may lead to genomic instability and facilitate tumor invasion and metastasis. Previously we have reported the involvement of oxidative stress in tumor progression or regression. We have shown that respiratory burst of granulocytes is significantly increased in tumor-bearing animals in an early stage of tumor development. Furthermore, we found that tumor progression is associated with the constant increase in the oxidative burst of granulocytes, whereas in animals with tumor regression the respiratory burst of granulocytes decreased to normal values following the tumor disappearance. ROS, present in the high levels in an early stage of tumor development, may consequently influence the induction of nuclear factor-kB (NF-κB) intracellular signaling repressing the Nrf2-ARE pathway at transcriptional level. NF-κB is ubiquitously expressed transcription factor mostly present in cytoplasm, bound to its inhibitor-kappa B (IκB). Activated NF-κB and its activating signaling pathways regulate cell adhesion, differentiation, growth, apoptosis, malignant transformation and inflammatory response. Granulocytes were shown to be the first cells that rapidly infiltrate the site of tumor transplantation thus supporting the possibility that granulocyte response to early stage of cancer is important for spontaneous regression. In the presence of tumor cells activated granulocytes produce higher amounts of ROS thus inhibiting the tumor cell proliferation. One of the mechanisms by which oxidative burst of granulocytes may lead to tumor destruction could be by influencing the Nrf2 signaling pathway (Fig.). Namely, tumor cells are more resistant to oxidative stress by the process of antioxidant systems activation. ROS produced by...
oxidative burst of granulocytes influence the NF-κB signaling pathway by repressing the Nrf2-ARE pathway and thus leading to malignant destruction. However, further increase in ROS levels may facilitate tumor promotion. Namely, polyunsaturated fatty acids that are esterified in membrane or storage lipids are subject to ROS-induced peroxidation resulting in the destruction of biomembranes. Final products of lipid peroxidation are reactive aldehydes among which is 4-hydroxy-2-nonenal (HNE), denoted the “second toxic messenger of free radicals”\textsuperscript{95-97}. Lipid-derived aldehydes are more stable than ROS and can therefore diffuse to targets far from the initial oxidative injury. Many deleterious effects have been attributed to HNE\textsuperscript{98} such as modulation of cell growth\textsuperscript{99,100}, differentiation\textsuperscript{101} and cell signaling\textsuperscript{102,103}. HNE is also very effective in binding to DNA or proteins leading to adduct formation, eliciting mutagenic or carcinogenic effects\textsuperscript{9,104}. Finally, HNE is also reported to have a role in Nrf2 signaling by inducing Nrf2 expression\textsuperscript{105}, which may further support proposed Nrf2 tumor promoting role\textsuperscript{79} in the later stages of cancer\textsuperscript{79}. However, in an early stage of tumor development ROS may be of crucial importance in repressing the Nrf2 pathway thus leading to tumor regression.

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**References**


49. Zhang DD, Lo SC, Cross JV, Templeton DJ, Hannink M. Direct evidence that sulfhydryl groups of Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. Mol Cell Biol 2003; 23 : 8137-51.


51. Stewart D, Killeen E, Naquin R, Alam S, Alam J. Degradation of Nrf2 by the 26 S proteasome.


56. Bloom DA, Jaiswal AK. Phosphorylation of Nrf2 at Ser40 by protein kinase C in response to antioxidants leads to the release of Nrf2 from Keap1, but is not required for Nrf2 stabilization/accumulation in the nucleus and transcriptional activation of antioxidant response element-mediated NAD(P)H:quinone oxidoreductase-1 gene expression. J Biol Chem 2003; 278 : 44675-82.


65. Lee JM, Moehlenkamp JD, Hanson JM, Johnson JA. Nrf2-dependent activation of the antioxidant responsive element by tert-butylhydroquinone is independent of oxidative stress in IMR-32 human neuroblastoma cells. Biochem Biophys Res Commun 2001; 280 : 286-92.


Liu GH, Qu J, Shen X. NF-kB/p65 antagonizes Nrf2-ARE pathway by depriving CBP from Nrf2 and facilitating recruitment of HDAC3 to MafK. Biochim Biophys Acta 2008; 1783 : 713-27.


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