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REACTIVE OXYGEN SPECIES IN TUMOR PROGRESSION

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TABLE OF CONTENTS

1. ABSTRACT

The generation of reactive oxygen radicals in mammalian cells profoundly affects numerous critical cellular functions, and the absence of efficient cellular detoxification mechanisms which remove these radicals can result in several human diseases. Growing evidence suggests that reactive oxygen species (ROS) within cells act as second messengers in intracellular signaling cascades which induce and maintain the oncogenic phenotype of cancer cells. ROS are tumorigenic by virtue of their ability to increase cell proliferation, survival, cellular migration, and also by inducing DNA damage leading to genetic lesions that initiate tumorigenicity and sustain subsequent tumor progression. However, it is also known that ROS can induce cellular senescence and cell death and can therefore function as anti-tumorigenic agents. Therefore, the mechanisms by which cells respond to reactive oxygen species depends on the molecular background of cell and tissues, the location of ROS production and the concentration of individual ROS species. Carcinoma cells produce ROS at elevated rates *in vitro*, and *in vivo* many tumors appear persistent to oxidative stress. Thus, the finding that a diet rich in antioxidants or the elimination of ROS by antioxidant compounds prevents the development of certain cancers provided the setting for subsequent investigation of the tumorigenic actions of reactive oxygen species. This review outlines the current knowledge on the various roles of ROS in tumor development and progression.

2. INTRODUCTION

Reactive oxygen species such as superoxide (O_2•) or its breakdown product hydrogen peroxide (H_2O_2) have been implicated in the development of several diseases such as diabetes, heart disease, mitochondrial disease, various neurodegenerative diseases and cancer. ROS play a major role in tumor initiation induced by a variety of agents both in animal models of disease and also in humans (1-3). For example, ROS are implicated in tumor induction mediated by phorbol esters, organic peroxides, heavy metals, asbestos, cigarette smoke and silica (4). In cancer cells, oxidative stress has been linked to the regulation of numerous cellular processes including DNA damage, proliferation, cellular adhesion and migration and the regulation of cell survival or death (5).

Initial experiments on the role of ROS in tumor initiation revealed that ROS act as DNA-damaging agents, effectively increasing the mutation rate within cells and thus promoting oncogenic transformation (6). Most of the DNA-damaging effects of ROS are due to hydroxyl radicals which possess the capacity to act in a non-specific, destructive manner. However, more recent studies have revealed that in addition to inducing such non-specific actions, ROS can specifically activate certain intracellular signaling cascades and thus contribute to tumor development and metastasis through the regulation of cellular phenotypes such as proliferation, death and motility (Figure 1). Growing evidence suggests that in many instances, the production of reactive oxygen species is tightly regulated and their downstream targets are quite specific (7). In the last few years, notable advances have been made in defining the roles of ROS as *bona-fide* second messengers (8), by identifying the specific signaling pathways that utilize intracellular oxidants as effector molecules and the signaling proteins which are their redox-dependent targets (Figure 2). These studies have significantly advanced our understanding of how reactive oxygen species participate in diverse processes such as transformation and motility leading to tumor progression.

Another aspect which has emerged in the field is how distinct reactive oxygen species affect different cellular responses. ROS are divided into free oxygen radicals and non-radical ROS. Free radicals contain one or more unpaired electrons and these include superoxide (O_2•), hydroxyl- (•OH), nitric oxide (•NO), alkoxyl- (RO•),

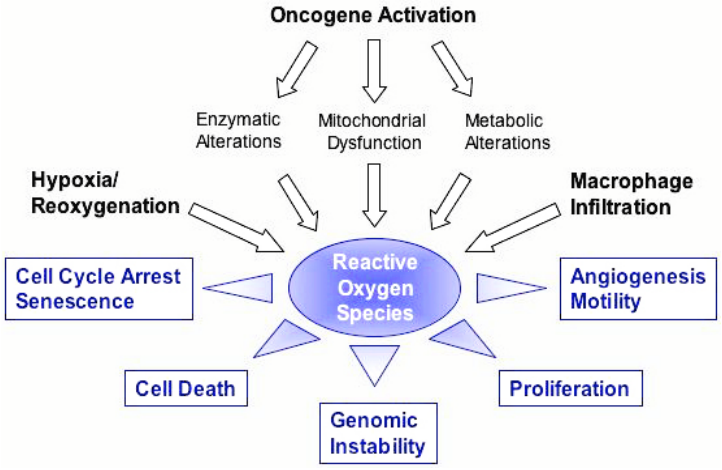

Figure 1. Scheme showing the generation and effects of reactive oxygen species in tumor progression. Oncogene activation, macrophage infiltration or hypoxia/reoxygenation in tumors induce the generation of reactive oxygen species (ROS). These ROS have roles in mediating cell proliferation, genomic instability, cell motility and angiogenesis and thus can contribute to tumorigenesis, but also can induce cell cycle arrest, senescence and cell death and thus attenuate tumor growth.

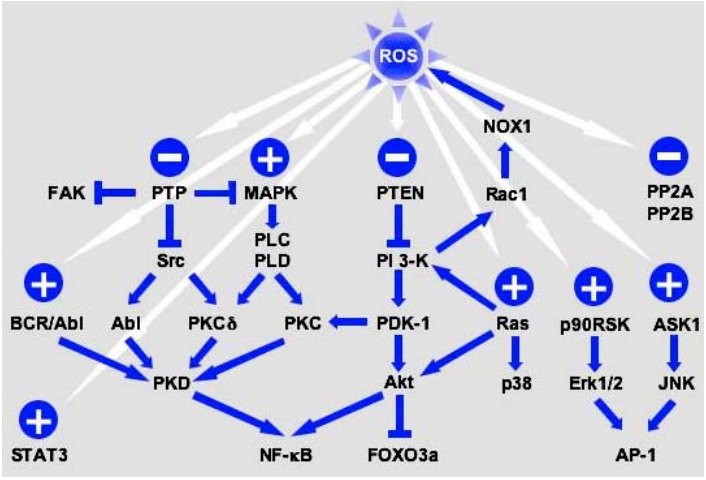

Figure 2. Reactive oxygen species-regulated signaling cascades. Reactive oxygen species inhibit phospho-serine/threonine-, phosphotyrosine- and phospholipid-phosphatases such as PP, PTP and PTEN, probably by directly regulating their active site cysteines. This leads to the upregulation of several signaling cascades, most prominent PI 3-Kinase-dependent, MAPK-dependent and Src/Abl kinase-dependent signaling pathways. These signaling cascades, as well as dimer formation due to inter-chain disulphide bridging mediated by oxidation (e.g. STAT3), lead to the activation of several redox-regulated transcription factors such as AP-1, FOXO3a, STAT3 and NF-κB.

or peroxyl- (ROO•) radicals. Non-radical ROS include hydrogen peroxide (H_2O_2), organic hydroperoxides (ROOH) and hypochloride (HOCl). Hydrogen peroxide is produced by a variety of intracellular reactions, particularly the oxidative electron transport chain in the mitochondria. H_2O_2 itself is not reactive toward DNA and most of its effects are due to the production of hydroxyl ions via the Fenton reaction (9). On the other hand, •OH is highly reactive towards DNA and is known to activate certain oncogenes such as K-Ras (10). Like hydrogen peroxide, most superoxide is generated by the mitochondrial electron transport chain (11). Superoxide-stimulated cellular damage is also due to •OH production via the Haber-Weiss reaction (12). •NO plays a role in the killing of tumor cells by activated macrophages (13) and reacts with superoxide to produce the peroxinitrite radical which is similar to •OH in its reactivity.

Reactive oxygen species can be generated within cells by a variety of enzymatic systems. A major source of ROS is aerobic respiration in the mitochondria. In tumor cells, oxygen radicals can also be generated by the NADPH-oxidase, thymidine phosphorylase (14) or by membrane receptors which stimulate mitochondrial ROS production (15). Superoxide, as with the majority of reactive oxygen species, undergoes rapid degradation and is

sequentially reduced to hydrogen peroxide and hydroxyl radicals which are then degraded to water. Thus, enzymes such as catalase and manganese superoxide dismutase (MnSOD) which catalyze these reactions play crucial roles in mediating cellular detoxification from ROS. Interestingly, in this context the antioxidants superoxide dismutase, glutathione peroxidase, catalase and malondialdehyde show altered expression or activity in tumor cell lines and tumor tissue (16). It is therefore not surprising that in the last few years it has become evident that antioxidants hold great promise as chemopreventitive agents which may useful in the treatment of cancer. In this regard, resveratrol (*trans*-3,4',5-trihydroxystilbene), a phenolic antioxidant found in human diet has been described to function as anti-tumor agent (17).

The understanding which species of ROS are generated, where they are released and how they control cellular signaling cascades leading to proliferation, cell death and motility is likely to provide new opportunities for pharmacological and genetic intervention to manipulate the transformation process.

3. REACTIVE OXYGEN SPECIES IN TUMOR GROWTH AND CELLULAR SURVIVAL

In vitro, human tumor cells produce ROS at a far greater rate than non-transformed cell lines, (14) and *in vivo*, constitutive oxidative stress has been detected in breast carcinoma (18). This effect has been proposed to be due to increased expression of NADPH-oxidases such as the Nox proteins which contribute to increased hydrogen peroxide levels, cell transformation and aggressive tumor initiation (19). Reactive oxygen species damage DNA and thus have mutagenic activity that promotes carcinoma initiation and progression. However, in a more specific manner, low concentrations of superoxide and hydrogen peroxide actually stimulate proliferation and enhanced survival in a wide variety of cell types (20-22). The prevention of apoptosis combined with DNA-damage, inefficient repair mechanisms and increased proliferation therefore increases the pool of genetically altered cells, which can give rise to transformed progeny. Another important factor in tumorigenesis is hypoxia/reoxygenation due to rapid outgrow of the blood supply.

Elevated oxidative stress has been found in many different types of cancer cells and the introduction of antioxidants can inhibit tumor cell proliferation, which points to an important role of ROS in mediating the loss of growth control (23). This may be due to the ROS-mediated activation of MAPK (Mitogen-Activated Protein Kinase) (24, 25). Aside from elevated ROS, the reduction in physiological tissue oxygen tension (hypoxia), which occurs during tumor initiation, can also regulate cancer cell proliferation. Under hypoxic conditions, cells undergo a shift from aerobic to anaerobic metabolism (26) and hypoxia induces several genes and transcription factors such as HIF-1α (Hypoxia Inducible Factor-1α), a key transcription factor in the hypoxic response (27). Limitations in oxygen supply to cells by prolonged hypoxia results in cell death. Since new blood vessels are aberrant or have poor blood flow, tumors often become hypoxic. Under hypoxic conditions cells activate signaling pathways which regulate proliferation, angiogenesis and death. Cancer cells have adapted these pathways effectively allowing tumors to survive and even grow under adverse hypoxic conditions. This adaptation of tumor cells to hypoxia contributes to the malignant phenotype and to aggressive tumor progression (27), and low oxygen tension in tumors is associated with increased metastasis and poor survival of patients in several squamous tumors (28, 29).

The reoxygenation of blood and the reperfusion of hypoxic tissue can increase the concentrations of free oxygen radicals (30). ROS produced by hypoxia/reoxygenation have damaging effects in cells, cause tissue injury, but also play a crucial role in vascular angiogenesis (31). Tumors rapidly outgrow their blood supply leading to glucose deprivation and hypoxia. Glucose deprivation depletes intracellular pyruvate, thus preventing the decomposition of endogenous oxygen radicals (32). In response to hypoxic conditions, breast carcinomas stimulate blood vessel development (angiogenesis). The blood flow within these new vessels is often chaotic and causes periods of hypoxia followed by reperfusion, which causes release of ROS (33). ROS can increase the production of the angiogenic factors IL-8 (Interleukin-8) and VEGF (Vascular Endothelial Growth Factor). Additionally, ROS promote the secretion of the matrix metalloprotease MMP-1 by tumor cells, which promotes vessel growth within the tumor microenvironment. Blood vessel growth within the tumor increases the risk of blood-borne metastases. Another way by which oxidative stress increases blood supply is by triggering vasodilatation. ROS can activate heme oxygenase-1, which creates carbon monoxide, or induce iNOS (inducible Nitric Oxide Synthase) whose product is nitric oxide (•NO). Both carbon monoxide and nitric oxide are vasodilatators (34).

In contrast to the role of ROS as mediators of cellular proliferation, angiogenesis and oncogenic transformation, oxidative stress has also been demonstrated to induce apoptosis, cell cycle arrest and cellular senescence. In apoptosis-inducing processes, ROS generation is attributed to the uncoupling of the electron transport chain in mitochondria and to a decrease in mitochondrial membrane potential. Mitochondria are both a source and target of ROS. How ROS act in this scenario to activate apoptotic signaling pathways is not entirely clear. Oxidation of mitochondrial pores by ROS may contribute to cytochrome C release due to the disruption of mitochondrial membrane potential (35). Other molecular mechanisms by which ROS act in a pro-apoptotic manner are through the activation of JNK (c-Jun N-terminal Kinase) or the transcription factor FOXO3a. Moreover, superoxide production by the Rac/NADPH oxidase pathway was recently shown to also function in a pro-apoptotic manner (36). Apoptosis in response to ROS is also dependent on the tumor suppressor p53. Constitutive oxidative stress in tumor cells might therefore lead to the selection of p53 deficient-clones and thus to apoptosis resistance.

ROS can be considered as mediators of cell death (by apoptosis or necrosis) since there are numerous examples of inhibition of apoptosis by antioxidant compounds. For example, the anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-XL have been shown to antagonize ROS production and to protect cells from ROS-mediated apoptosis (37, 38). It is also known that increased expression of antioxidant proteins such as peroxiredoxin III and catalase can prevent hydrogen peroxide-induced apoptosis in tumor cells (39). However, studies have also revealed that ROS can activate anti-apoptotic pathways leading to increased cellular survival. As an example, although nitric oxide (NO•) mediates apoptosis in many cell types (40), in some cell types it actually functions as a survival factor by inducing heat shock proteins such as Hsp27 which elevate glutathione, an intracellular antioxidant (41). Moreover, NO• is involved in host-anti-cancer defense, exerted by macrophages and NK (natural killer) cells. The main target for NO• is probably the mitochondria, since apoptosis is blocked by expression of Bcl-2 (42). These seemingly contradictory observations can be explained if actual concentrations of ROS determine if apoptosis is induced or if it is inhibited. Alternatively, the intracellular localization of ROS production may also provide an explanation as to whether oxygen radicals act in a pro-or anti-apoptotic manner.

ROS can mediate cellular senescence in response to mitogenic signals (43), and ROS are linked to the development of a senescent phenotype in response to growth factor-activated signaling pathways. Peroxides have been found to initiate cell cycle checkpoint arrest in several eukaryotic cell types by suppressing S-phase entry via a senescent-like growth arrest in G1. This is accompanied by the up-regulation of the tumor suppressor p53 and its transcriptional target p21, which are known to regulate the G1 checkpoint (44). Moreover, H_2O_2 also has been described to regulate S-phase and G2-phase checkpoints (45).

Thus, besides their function in tumor growth and angiogenesis, ROS can also be considered as mediators of stress signaling cascades which suppress cellular transformation by inducing cell cycle arrest, apoptosis and cellular senescence.

4. REACTIVE OXYGEN SPECIES IN CELL MOTILITY AND METASTASIS FORMATION

In addition to regulating tumor growth and survival, ROS also control mechanisms which are associated with the formation of tumor metastases. The cellular processes which are linked to this function, are decreased cell adhesion to the basal lamina and increased migratory and invasive potential which permit cancer cells to enter the vasculature. In this context, increasing blood vessel growth due to hypoxia and reoxygenation increases the risk of blood-borne metastasis. Moreover, migrating cells also have an increased propensity to escape from detachment-induced cell death (anoikis) and thus induce the metastatic phenotype.

When exposed to oxidative stress, certain carcinoma cells exhibit decreased attachment to the basal lamina (46). This suggests that tumors cells subjected to oxidative stress might easily detach and more efficiently enter blood vessels. In this scenario, oxidative stress may aid the seeding of metastatic tumor cells (46). Reactive oxygen species can regulate cellular adhesion by modulating integrin function, for example by regulating integrin-transduced signals. In this context, in endothelial cells the crosslinking of integrins induces MAPK, PI 3-K (phosphoinositide 3-kinase) and NF-κB (Nuclear Factor-κB) activation pathways, which are also regulated by oxidative stress (47). Recent data point to a role for the small GTPase Rac1 in motility and invasion of tumor cells *in vitro* by altering cell-cell and cell-matrix adhesion. For example, Rac1 activity induces ROS production in endothelial cells. These ROS can mediate Rac1-induced loss of cell-cell adhesion in primary human endothelial cells and thus might loosen the integrity of the endothelium (48). Rac1 also plays a role in ROS-mediated actin cytoskeleton reorganization (49). In addition to Rac1, p38 MAPK (24) and phosphorylation of Hsp27 (Heat shock protein 27) by p38 have been shown to induce changes in actin dynamics (50). For example, Hsp27 promotes the migration of some breast cancer cell lines on laminin (51). Moreover, the tyrosine kinase FAK (Focal Adhesion Kinase) has been shown to be the initiator of focal adhesion formation in adherent cells, leading to cell spreading, cell migration, proliferation and prevention of death from apoptosis. ROS have been shown to induce tyrosine phosphorylation of FAK leading to its membrane localization, an important feature of FAK function critical for its biological function (52).

Oxygen radicals may augment tumor invasion and metastasis by increasing the rates of cell migration. Treatment of mammalian carcinoma cells with hydrogen peroxide prior to intravenous injection into mice enhances lung metastasis formation, indicating that an important function for ROS is the seeding of metastatic tumor cells (46). This might be due to a decreased attachment of tumor cell to the basal lamina, or alternatively be due to the increased activity or expression of proteins which regulate cellular motility. For instance, oxidative stress regulates the expression of the cell surface protein ICAM-1 (Intercellular Adhesion Protein-1, CD54) in endothelial and epithelial cells, most likely due to the activation of NF-κB. ICAM-1 together with IL-8 (Interleukin-8) regulates the trans-endothelial migration of neutrophils and has a potential function in tumor metastasis (53).

A separate mechanism by which oxidative stress may induce tumor cell migration and invasion is through the activation or upregulation of MMPs (Matrix Metalloproteinases) or the inactivation of TIMPs (Tissue Inhibitors of Metalloproteinases) by oxidative stress. Increased activation of MMPs correlates with increased cell motility, tumor invasion and metastasis, since MMPs are capable of cleaving most components of the basement membrane and extracellular matrix (54). For example, MMP-2 is a gelatinase that play a major role in the invasion

and metastasis of some cancers (55, 56). High levels of MMP-2 correlate with poor prognosis of breast cancer patients and active MMP-2 is detected more frequently in malignant than in benign breast tumors (33). The activation of MMPs, such as MMP-2, probably occurs by the reaction of oxygen radicals with thiol groups in the protease catalytic domain (56). MMPs are secreted in a latent zymogen form in which the pro-domain shields the catalytically active form. Redox-dependent MMP activation leads to the cleavage of this pro-domain, which is either mediated by an auto-activation mechanism or by other proteases (56, 61).

Additionally to their role as key regulators of MMP activation, ROS have been implicated and MMP gene expression (57). In addition to hydrogen peroxide, nitric oxide donors as well as increased expression of the inducible nitric oxide synthase (iNOS) stimulates the expression of several MMPs (MMP-1, MMP-3, MMP-9, MMP-10, MMP-13) (57). Similarly, UV irradiation which is known to exert damaging effect via ROS generation, induces MMP expression such as the interstitial collagenase MMP-1 and stromelysin-1 (MMP-3) in human dermal fibroblasts (58). MMP-1 expression is also elevated by agents which decrease the detoxifying capacity of cells, such as the glutathione synthesis inhibitor BSO (buthionine sulfoximine) or the catalase inhibitor aminotriazole (59). This is consistent with the inhibition of MMPs expression with antioxidants such as N-acetylcysteine or catalase (59, 60).

Oxidative stress may also modulate MMP expression by activation of Ras, or direct activation of the MAPK family members ERK1/2 (Extracellular-signal Regulated Kinase 1/2), p38 and JNK, or inactivation of phosphatases which regulate these proteins. Ras can be activated by ROS via oxidative modification of its Cys118 residue, which leads to the inhibition of GDP-GTP exchange (62). Induction of various MMPs (MMP-1, MMP-2, MMP-3, MMP-7, MMP-9) has been shown to occur in response to expression of the Ras oncogene in cultured cells (63). The direct activation of the Ras effector ERK1/2 leads to the induction of the Ets transcription factor and JNK-regulated Jun and Fos transcription factors which form the AP-1 (Activator Protein-1) complex. Both AP-1 and Ets regulate MMP expression (57, 64). Further, other MAPKs such as p38 also seem to have a function in regulating the expression of several MMPs (MMP-1, MMP-3, MMP-9, MMP-13) (64). However, MMP expression is regulated by complex mechanisms and not just Ras-ERK signaling. For example, other redox-sensitive transcription factors such as NF-κB have been implicated in MMP gene regulation (57). Finally, oxidative stress may also regulate tumor cell motility by inactivating protease inhibitors. For example, it has been shown that inhibitors such as plasminogen activator inhibitor, which is implicated in tumor metastasis, can be inactivated by oxidation of methionine residues at its active site (14, 65).

It is also worth recognizing that ROS may also promote tumor metastasis by increasing vascular permeability, either by direct damage to endothelial cells or by the upregulation of iNOS or heme oxygenase-1 (33). For example, low concentrations of superoxide and hydrogen peroxide stimulate endothelial migration and tube formation in an *in vitro* model of angiogenesis (66).

5. GENERATION OF REACTIVE OXYGEN SPECIES AND DETOXIFICATION MECHANISMS

The number of possible sources for oxidative stress is large and includes mitochondrial oxidative phosphorylation, ionizing radiation (IR), cytokine or growth factor receptor-induced peroxide responses, inflammation (particularly chronic inflammation) and pathological metabolic processes (45, 67). In the face of the deleterious effects of reactive oxygen species, aerobic organisms have developed a wide array of different mechanisms to reduce oxidative stress. These mechanisms include constitutive and inducible antioxidants such as catalase and superoxide dismutases as well as DNA repair enzymes and proteins which control the cell cycle.

The generation of superoxide radicals by activated neutrophils or macrophages as a mechanism to kill bacteria or cells was the first evidence which pointed to the importance of reactive oxygen species in cellular responses. Macrophages generate superoxide via the NADPH-oxidase, located at the plasma membrane. It rapidly became apparent that this oxidative burst is also inducible by growth factors and cytokines (68). The generation of ROS in non-phagocytic cells has been shown to be induced in response to various stimuli such as TNF-α (Tumor Necrosis Factor-α), although recent studies have revealed that tumor cells also use NADPH-oxidase to produce oxygen radicals (14, 69). In addition to intracellular release of superoxide, tumors can also be infiltrated by macrophages which may contribute to oxidative stress either through induction of the NADPH-oxidase burst, or through TNF-α secretion (46). Other intracellular pathways which stimulate ROS production include p66shc, a protein which up-regulates intracellular oxidant levels in mammalian cells (70). Interestingly, elevated p66shc levels are found in breast cancer cell lines and in primary tumors with high metastatic potential (71). Another enzyme overexpressed in majority of breast carcinomas and involved in the induction of ROS is thymidine phosphorylase which catabolizes thymidine to thymine and 2-deoxy-D-ribose-1-phosphate. 2-deoxy-D-ribose-1-phosphate is a reducing sugar which glycates proteins and effectively generates oxygen radicals (33).

As already mentioned, one major source of oxygen radicals is aerobic respiration performed by the mitochondria. Oxidation processes play a crucial role in ATP synthesis by the mitochondria. The production of ROS in mitochondria occurs as a side reaction of the electron transport chain, and is dependent on the partial oxygen pressure in the mitochondrial environment. Saturation of the mitochondrial respiratory chain terminal oxidase results in very rapid increase in superoxide production (72). Most of the superoxide radicals generated at the mitochondria are degraded to hydrogen peroxide by the manganese superoxide dismutase (MnSOD) in the mitochondrial matrix. Aside from the mitochondria,

peroxisomes are one of the other main sites where free oxygen radicals are generated and also scavenged (73). Peroxisomes are subcellular respiratory organelles which have the potential to carry out metabolic pathways different from those in mitochondria. Superoxide is produced in the peroxisomal matrix (mediated by xanthine oxidase) and in peroxisomal membranes (74, 75). Peroxisomes use flavin oxidase or copper/zinc superoxide dismutase (Cu/Zn SOD) to hydrolyze superoxide anions and catalase or peroxiredoxin (Prx) for further degradation.

Superoxide can lead to cellular damage by reacting with nitric oxide to generate peroxinitrite anions, which cause DNA damage and lipid peroxidation (76). The majority of superoxide undergoes rapid degradation to oxygen and hydrogen peroxide, a reaction, which can either be spontaneous or be regulated (77, 78). The most important enzymatic systems for superoxide scavenging are MnSOD, Cu/Zn SOD and glutahione (GSH; gamma-glutamylcysteinylglycine). Superoxide dismutases use superoxide as a substrate to generate hydrogen peroxide. Hydrogen peroxide is then reduced to water by catalase and peroxiredoxins. Peroxiredoxins have peroxidase activity and reduce peroxynitrite and peroxides (H_2O_2, ROOH,) to ROH and water. They are ubiquitous enzymes and localized in the cytosol, mitochondria, nuclei and peroxisomes of mammalian cells. Thus far, six isoforms have been identified in mammalian cells (PrxI-PrxVI) (79).

Glutathione acts as a radical scavenging antioxidant and is oxidized by superoxide to a glutathionyl radical which has a potentially damaging effect by initiating lipid peroxidation or the abstraction of hydrogen from peptides or carbohydrates (78, 80-83). Glutathione also functions as a co-factor for the glutathione peroxidase (Gpx), where it provides two electrons for the reduction of hydrogen peroxide to water (78).

The antioxidants superoxide dismutase, glutathione peroxidase, catalase, peroxiredoxin and malondialdehyde show altered expression or activity in tumor cell lines and tumor tissue (16). The exact role of these antioxidants in tumor progression is not clear. For example, peroxiredoxins have been described to control cytokine-induced peroxide levels, and thus regulate cell proliferation and apoptosis. It was also shown that PrxI has an essential role in tumor suppression, since mice lacking this enzyme have a shortened lifespan due to the development of several malignant cancers (84). Moreover, the down-regulation of another peroxiredoxin, PrxV, can stimulate the formation of etoposide-induced double stranded DNA breaks (85). Studies have also pointed to a role for peroxiredoxins in tumor progression. For example, in lung cancer tissues and cells elevated PrxI expression has been demonstrated (86), and it has been shown that PrxI plays a role in tumor metastasis (87). Similarly, increased expression levels of the peroxiredoxins PrxII, PrxIV and PrxV have been detected in breast carcinomas (88), PrxII levels are elevated in mature endothelial cells of benign vascular tumors of the skin (89) and PrxIII levels are elevated in hepatocellular carcinomas (90). Increased expression of mitochondrial PrxIII protects cancer cells against hypoxia and drug-induced hydrogen peroxide-dependent apoptosis (91). Thus, peroxiredoxins may induce redox changes in cancer cells by detoxifying hydrogen peroxide, and therefore affect tumor cell proliferation and malignant progression.

6. CELLULAR SIGNALING MEDIATED BY REACTIVE OXYGEN SPECIES

Many studies have shown that ROS production occurs as a result of activated growth factor receptor signaling. Examples include receptor-tyrosine kinases such as PDGF and EGF (15, 92-94), cytokine receptors such as TNF-α and IFNγ (94, 95) or interleukin receptors (96). It is generally accepted that ROS generated by these ligand/receptor-initiated pathways can function as true second messengers and mediate important cellular functions such as proliferation and programmed cell death (23, 25). An overview of ROS-regulated signaling cascades is presented in Figure 2.

Receptor tyrosine kinases (RTK) can induce ROS formation via phosphoinositide 3-kinase (PI 3-K), which is activated directly by binding to the receptor and by coupling to GTP-bound, activated Ras (97, 98). Once activated, PI 3-K activates the small GTP-binding protein Rac1 via the Sos1/Eps8/E3b1 complex (23). The small GTPase Rac1 is a key effector in ROS-generating pathways. In transformed fibroblasts, Rac1 is directly linked to the production of superoxide anions and to the transformation of cells caused by ROS production (25). Rac1 causes ROS production by activating the NADPH-oxidase Nox1 (92). In turn, Nox1 is capable of transforming cells, and these cells produce aggressive tumors when injected into nude mice (99). It was recently shown that increased levels of hydrogen peroxide mediated by Nox1 are required to maintain the transformed state (19). A subset of the nerve growth factor (NGF)/TNF-α receptor family, known as death receptors, contain a so-called death domain which induces apoptotic cell death via caspase-dependent mechanisms (100, 101). These death receptors seem to induce ROS production at the mitochondria, which in turn is necessary for the activation of caspases (102, 103). Moreover, TRAF4, a component of the TNF-α signaling pathway, binds directly to the NADPH-oxidase complex and provides a link between ROS generation and death-receptor signal transduction (104). Notably, TNF-α–induced ROS production can also activate anti-apoptotic pathways by stimulating the NF-κB transcription factor, leading to induction of antioxidant genes such as MnSOD and catalase (105).

Aside Rac1, other PI 3-K targets involved in anti-apoptotic signaling pathways such as PDK-1 (3'-phosphoinositide-dependent kinase-1) and Akt/PKB (Protein Kinase B) are also activated by reactive oxygen species (106-109). PDK-1 activates Akt/PKB (109, 110) and Akt/PKB has been shown to regulate cellular survival by the activation of NF-κB and the inhibition of Forkhead transcription factors such as FOXO3a (70, 111).

ROS also activate members of the MAPK family such as p38, JNK, and ERKs (80, 81, 112-115). For instance, superoxide and hydrogen peroxide, products of Nox1 activity, can activate the MAPK cascade at the level of MEK and ERK1/2 (24). However, although transformed cells produce elevated levels of ROS (14), it has also been suggested that ROS production sensitizes oncogenically-transformed cells to genotoxic agents by activating the MAP kinases p38 and JNK (116).

Several PKC isoforms are activated by ROS (117, 118). The mechanisms which lead to activation of PKCs in response to ROS production have been explored. Though the precise mechanism can be different depending on the isoform in question, one generic mechanism is tyrosine phosphorylation of PKCs mediated by tyrosine kinases Src and/or Abl. However, since oxidative stress increases the activity of receptor-regulated phospholipases such as PLC, PLA2 and PLD (119-121), DAG (diacylglycerol)-mediated activation of PKC isoforms in response to oxidative stress also likely plays an important role. It remains to be established if the activation of PKCs by canonical DAG release compared to ROS-stimulated tyrosine phosphorylation results in altered substrate specificity or cellular function. However, what is known is that PKC activation plays a critical role in cancer cell proliferation and clearly this has important functional consequences for redox-activated signaling downstream of PKC, for example, increased expression of the *c-jun* and *c-fos* genes.

The finding that in Ras transformed fibroblasts ROS drive cell cycle progression without the activation of MAP kinases suggests that additional mechanisms or signaling cascades must exist to sense oxidative stress (25). One recently described example is a signaling pathway regulated by PKD (Protein Kinase D). In response to oxidative stress, PKD is activated in a PI 3-K and Akt/PKB-independent manner and regulates a cellular survival pathway which induces NF-κB (122-128). PKD has been shown to be localized in invadopodia at sites of extracellular matrix degradation and to be downregulated in prostate cancer (129, 130). PKD is a sensor for oxidative stress and its kinase activity is tightly regulated by phosphorylation, mediated by a Src/Abl signaling pathway and by protein kinase Cδ (PKCδ) (124, 128, 131-133). These events are required for full activation of PKD and subsequent regulation of NF-κB in response to exposure of cells to ROS. With respect to apoptotic mechanisms in tumor cells, it is interesting to note that that PKD and its upstream activators translocate to the mitochondria (132, 133), which are known to generate ROS in response to a number of environmental insults.

The activation of tyrosine kinases by oxygen radicals most likely occurs via the inhibition of phosphatases. H_2O_2 is a mild oxidant that can oxidize cysteine residues in proteins. Protein tyrosine phosphatases (PTPs) contain an essential cysteine residue in the signature active site which is important for the catalytic function. Thus, PTPs undergo H_2O_2-mediated inactivation due to the oxidation of these important cysteine residues (134-136). Also, protein serine/threonine phosphatases such as PP2A and PP2B/calcineurin are redox-regulated and PP2B/calcineurin is inhibited by hydrogen peroxide and superoxide (137, 138). Another phosphatase that contains an active site cysteine, the lipid phosphatase PTEN and the cell cycle regulatory enzyme Cdc25C have also recently been shown to be redox-regulated (139, 140). Cysteine oxidation mediated by oxygen radicals probably also contributes to the direct activation of tyrosine kinases such as RTKs, Abl and Src, though the precise mechanism is unclear (67). The tyrosine kinase Abl has been shown to be regulated by oxidative stress and to regulate cell shape and motility (141). In addition, *v-Abl* has a high oncogenic potential (142, 143). In response to exogenous oxidative stress, Abl translocates to the mitochondria where it associates with PKCδ (131, 132, 144). c-Abl also regulates glutathione peroxidase I (Gpx1) and catalase. Depending on cellular ROS concentration, both c-Abl and the related kinase Arg associate with and phosphorylate Gpx1, stimulating Gpx1 activity (145, 146). The tyrosine kinase Src has been shown to be regulated by ROS and to have a role in actin organization and cellular adhesion and motility (147, 148).

NF-κB is a redox-regulated transcription factor and a sensor for oxidative stress (149). It is activated by low concentrations of hydrogen peroxide and various antioxidants prevent its activation by cellular stimuli (150). However, it should be noted that the use of 'so called' antioxidant compounds can be misleading since they can non-specifically affect other targets and lower, for example, TNF/TR55 affinity or inhibit the activity of the IκB-ubiquitin ligase in an ROS-independent manner (151). Despite this, NF-κB is clearly important for tumor progression as demonstrated by recent studies which have associated NF-κB with oncogenesis and cellular adhesion in certain animal models of cancers (152). Several members of the NF-κB or IκB families derive from genes which are amplified or translocated in human cancers (153). However, the *v-Rel* oncogene is the only NF-κB-related protein that can directly transform cells *in vitro* and *in vivo* (154). NF-κB activation pathways associated with cancer are uncoupled from their normal modes of regulation, and in cancer cells a constitutive NF-κB activation has been demonstrated. For example, BCR-Abl transformed cells show constitutive NF-κB activity, and Hodgkin disease has been associated with a mutation in the gene which encodes IκBα (155). Thus, NF-κB has been implicated in the development of leukemia and lymphoma, which are both caused by uncontrolled proliferation of blood cells, as well as the development of cancers of epithelial origin (carcinomas) such as breast carcinoma, or gastric and colorectal carcinoma (reviewed in (152, 156)). Moreover, NF-κB can induce many of the genetic alterations which are required to render cells tumorigenesic. For example, NF-κB can stimulate cell proliferation by activating growth stimulatory target genes such as IL2, GM-CSF and CD40L (152), regulate the cell cycle through G1 cyclins or c-Myc (157-159), inhibit apoptosis (160-162) and attenuate the apoptotic response to genotoxic anti-cancer drugs and ionizing radiation (163, 164). The

regulation of NF-κB has been extensively studied during the last decade mostly by analyzing cytokine-mediated activation mechanisms (165-167). However, oxidative stress can activate this transcription factor through the activation of a variety of distinct signaling pathways (168-170). Our own studies have pointed to an important role for the serine/threonine kinase PKD in the activation of NF-κB via the canonical IKK (IκBα Kinase) complex in cells exposed to H_2O_2 (see above) (124). Others have shown that in certain cell types, oxidative stress-mediated activation of NF-κB occurs in an IKK-independent manner and through the tyrosine phosphorylation of IκBα (171, 172).

NF-κB target genes include anti-apoptotic proteins such as cIAPs, A20 or antioxidants such as MnSOD (173). NF-κB almost always drives transcription in association with other transcription factors (156). ROS and other free radicals can activate AP-1 and NF-κB coordinately and inhibition of both can abrogate transformation in some cell types (174). The active AP-1 complex is composed of the transcription factors c-Jun and c-Fos, which play important roles in proliferative responses (175). In oxidative stress signaling the hyperphosphorylation of MAP kinases such as JNK and p38 leads to AP-1 activation (176).

Besides NF-κB and AP-1, oxidative stress has been shown to increase the activity of a variety of other transcription factors such as FOXO3a, Gadd153/CHOP and STAT3 (126, 177-182). In some cases, transcription factor activation is due to increased phosphorylation by upstream kinases induced by oxidative stress (124, 178), whereas in other cases, oxidants may directly or indirectly modulate the redox status of critical cysteine residues that regulate DNA binding activity (179). For example, STAT3 (Signal Transducer and Activator of Transcription 3) which is persistently activated in almost all head and neck cancers, in multiple myeloma, lymphoma, leukemia and hepatocellular carcinoma, is activated by oxidative stress via dimer formation due to inter-chain disulphide bridging (183). Notably, the introduction of dominant-negative alleles of STAT3 into cells derived from either of these tumors results in increased apoptosis (reviewed in (156)). Recently, it was also demonstrated that the Forkhead transcription factor FOXO3a is regulated by exposure of cells to extracellular H_2O_2 (70, 184). In quiescent cells, FOXO transcription factors are predominantly nuclear and H_2O_2 has been described to promote nuclear export (70). Others have reported that exposure of cells to high doses of extracellular H2O2 causes activation of FOXO3a, which translates into sod2 (the gene which encodes MnSOD) induction and protection from death by apoptosis (177). Under these conditions, Akt/PKB, which in growing cells inhibits FOXO3a, is not active. FOXO3a also regulates GADD45, a growth arrest and DNA-damage inducible gene which mediates part of FOXO3a's effects on DNA repair (185).

Although an important role for FOXO3a in regulating cell growth and tumorigenesis has been described (186), the role of this transcription factor in tumor metastasis has not been explored. With respect to *sod2* gene regulation, it was recently demonstrated that expression of the *c-myc* gene in normal human fibroblasts raises the levels of intracellular ROS and that this is sufficient to induce DNA damage (187). Additionally c-Myc expression also represses *sod2* gene expression and targets peroxiredoxin3, thus changing the mitochondrial scavenging capacity (136, 188).

7. TARGETING REACTIVE OXYGEN SPECIES IN CANCER

Oxygen radicals are potent DNA-damaging agents which cause DNA strand breaks, alterations in purine and pyrimidine bases and sister chromatid exchanges. This genetic instability could lead to the inactivation of tumor suppressor genes or the activation of oncogenes and thus increase the malignant potential of the tumor. On the other hand, oxygen radicals induce cell death and many anticancer therapies such as the chemotherapeutic agents doxorubicin, etoposide and cisplatin or radiotherapy and photodynamic therapy lead to the generation of superoxide and oxygen radicals within carcinoma cells (33, 189). Persistent oxidative stress within carcinomas may cause resistance to these drugs by upregulation of antioxidant defenses and thus resistance to therapy. Thus, the exclusive use of these agents could be a double-edged sword since depending on their concentration, ROS may induce tumor progression or cell death.

Natural antioxidants such as vitamin A (retinoids), vitamin C (ascorbic acid), vitamin E (tocopherols), selenium and carotenoids have been implicated in the prevention of cancer and oxidative stress-related diseases. For example, β-carotene and retinoids may function as anticarcinogenic agents by antagonizing the biological effects of pro-oxidants on PKC (190). Recently, resveratrol (*trans*-3,4',5-trihydroxystilbene), a phenolic antioxidant found in grapes and other human dietary foods has been shown to function as a cancer preventive agent. Resveratrol and other plant polyphenols can activate sirtuins such as SIRT1, a family of NAD+-dependent protein deacetylases which can increase DNA stability (191, 192). The proliferation of various human malignant cell lines is decelerated by resveratrol, and resveratrol has been described to prevent TPA-mediated mouse skin tumor formation. At the cellular level, resveratrol regulates apoptosis and induces a cell cycle delay or accumulation of cells in S and G2 phase (193-195). Resveratrol-mediated regulation of apoptosis occurs most likely by the suppression of NF-κB (196). NF-κB is implicated in cancer development by increasing cellular survival. One mechanism by which resveratrol inhibits NF-κB includes the decreased phosphorylation of IκBα by the IKK complex. Resveratrol also suppresses the activation of signaling kinases such as PKCs and PKD (197-199). Both are oxidative stress and phorbol ester-activated enzymes which have been linked to cancer development and NF-κB activation (197, 198, 200). It has also been shown that resveratrol suppresses the transcriptional activation of cytochrome P-450 1A1 and that it inhibits the enzymatic activities of COX-1 and COX-2 (201).

Taken together, natural antioxidants such as resveratrol hold great promise as chemopreventive agents useful for treatment of cancer.

8. PERSPECTIVE

In the last few years, the importance of reactive oxygen species in the regulation of cellular processes controlling tumor formation has come center stage. A growing body of evidence suggests that ROS, aside from DNA-damaging function, can act as second messengers and control various signaling cascades which induce and maintain the oncogenic phenotype of cancer cells. The cellular signaling mechanisms activated by ROS are becoming clearer as is the contribution of several protein kinases in mediating ROS responses in tumor cells. Since a variety of transcription factors activated by oxidative stress are regulated by these signaling cascades, one future goal will be to identify the transcriptional program induced by these pathways and to ascribe functions of individual genes to oxidative stress-regulated diseases, such as aging and cancer.

The identification of ROS-stimulated signaling cascades and the finding that the elimination of excessive ROS by chemical or enzymatic antioxidants decreases the tumorigenicity of various types of tumor have opened up new areas of research for cancer cell biologists and pharmaceutical companies. More distant goals will likely focus on the development of inhibitors as well as activators of these signaling cascades to manipulate their action in aggressive human diseases in which the ROS generation occurs and where detoxifying pathways are often deregulated. Potential candidates are ROS-generating enzymes such as iNOS, Rac1 or enzymes which act as antioxidants such as MnSOD or catalase. Furthermore, small chemical antioxidants such as resveratrol hold particular promise as they are normal nutritional components and may provide the long-sought after treatments to halt or even prevent tumor development or progression.

However, it should be recognized that for efficient targeting of ROS in tumor therapy, it will be important to determine which specific oxygen radicals function in which aspect of tumor progression. For example, ROS such as •NO or H_2O_2 seem to play a key role in inducing tumor proliferation and promoting survival, albeit at certain doses. Yet at higher doses, they actually induce cell death. Thus, a therapeutically-induced increase in antoxidants may reduce tumor proliferation and metastasis formation, however, it also may protect these cells from undergoing apoptosis in response to chemotherapy.

Taken together, recent exciting advances in the field have provided a greater understanding of the role of ROS as mediators of tumor development. Experimental and epidemiological evidence indicates that the intracellular production of reactive oxygen species has a fundamental role in the initiation and promotion of carcinogenesis. There is therefore the realistic prospect for treatments aimed to reduce these ROS which could be curative against cancer proliferation or metastasis. Clearly, however, a more detailed understanding of ROS-mediated signaling pathways in tumor cells will be necessary to develop new strategies for therapeutic interventions.

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