[Frontiers in Bioscience, Landmark, 21, 839-855, January 1, 2016]

Pathophysiology of neutrophil-mediated extracellular redox reactions

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1. ABSTRACT

Neutrophil granulocyte leukocytes (neutrophils) play fundamental role in the innate immune response. In the presence of adequate stimuli, neutrophils release excessive amount of reactive oxygen species (ROS) that may induce cell and tissue injury. Oxidative burst of neutrophils acts as a double-edged sword. It may contribute to the pathology of atherosclerosis and brain injury but is also necessary in resolving infections. Moreover, neutrophil-derived ROS may also have both a tumor promoting and tumor suppressing role. ROS have a specific activities and diffusion distance, which is related to their short lifetime. Therefore, the manner in which ROS will act depends on the cells targeted and the intra- and extracellular levels of individual ROS, which can further cause production of reactive aldehydes like 4-hydroxynonenal (HNE) that act as a second messengers of ROS. In this review we discuss the influence of neutrophil mediated extracellular redox reactions in ischemia reperfusion injury, transplant rejection and chronic diseases (atherosclerosis, inflammatory bowel diseases and cancer). At the end a brief overview of cellular mechanisms to maintain ROS homeostasis is given.

2. INTRODUCTION

In 1882 a Russian scientist, Elie Metchnikoff**,** discovered phagocytosis, a cell-mediated immune response to foreign matter, based on “professional phagocytes” engulfing and destroying microorganisms. The discovery of Metchnikoff was acknowledged by the Nobel Prize in 1908. Although phagocytosis is an ancient cellular function, professional phagocytes have evolved only in higher organisms. Neutrophils, the most abundant white blood cells, are one of the professional phagocytes in humans and play a fundamental role in the innate immune response (1). It is well known that in the response to inflammatory stimuli, activated neutrophils adhere to activated vascular endothelium, leave the circulation, and migrate towards inflammatory foci. This multi-step process has recently been updated by Ley and his colleagues (2). During this process neutrophils release a wide variety of mediators that can promote lesion formation and progression, extracellular matrix degradation, and plaque erosion. At the site of inflammation the apoptosis of neutrophils and their clearance is necessary for the resolution of the infection (3).

The beneficial role of neutrophils as key players in host defense against microorganisms is well known. In addition to neutrophil participation in the killing of microorganisms, the involvement of neutrophils in immune reactions against cancer was also suggested; however the mechanisms by which neutrophils mediate immune response against cancer are not well understood.

The role of neutrophils in tumor development has been controversial and has received little attention; therefore in this review one section will be dedicated to neutrophil mediated malignant destruction.

Moreover, extended neutrophil accumulation can induce damage to the surrounding tissue and in this way contribute to the pathogenesis of disease (4). In addition, systemic inflammatory response increases oxidative activity of circulating neutrophils and may cause damage to initially unaffected organs. Increased oxidative activity of neutrophils could disturb oxidative homeostasis leading to the state of oxidative stress. The first defense line of individual cells from oxidative stress represents the plasma membrane redox system (PMRS). PMRS is an electron transport chain in the plasma membrane that provides electrons for energy metabolism and recycling of antioxidants. PMRS regulates numerous aspects of cell physiology and signaling, such as control of apoptosis induced externally, by mild oxidative stress (5). Beside PMRS, there are also other various redox systems (e.g. pyridine nucleotide redox system, glutathione and thioredoxin redox system) that participate in cell signaling and modulation of cell function (6,7).

In this review we will discuss the influence of neutrophils mediated extracellular redox reactions in various disorders, like ischemia reperfusion injury, transplant rejection and atherosclerosis. Also we will describe a potentially beneficial role of neutrophils in cancer regression. Finally, a brief overview of cellular antioxidant defense systems is given.

3. OXIDATIVE BURST OF NEUTROPHILS

In an infected tissue neutrophils engulf and destroy microbes with a combination of various microbicidal systems. NADPH oxidase dependent generation of superoxide anion ($^{\bullet}O_2^-$) and release of microbicidal proteins from preformed granules are the crucial killing mechanisms of neutrophils. Neutrophils employ NADPH oxidase to generate reactive oxygen species (ROS) inside the so-called phagosome in response to various microorganisms. However, in the response to soluble agonists neutrophils can also generate extracellular ROS.

Namely, the activation process of neutrophils is accompanied by the intense production of ROS (1,8), and an extended release of destructive hydrolytic enzymes (9). Oxidative burst of neutrophils is shown on Figure 1. Neutrophils generate extracellular superoxide anions by action of the membrane bound enzyme NADPH oxidases (Nox). Nox2 is the best- known member of the Nox family and is highly expressed in phagocytic cells. This is a complex flavoprotein, the activation of which is controlled by surface receptors. After activation of receptors, assembly of cytosolic activating proteins, $p47^{phox}$, $p67^{phox}$ (phox for phagocyte oxidase), and the small G protein Rac (1 or 2), occurs with the membrane-bound flavocytochrome b_{558} (10,11). The catalytic activity is achieved by the glycozylated $gp91^{phox}$ subunit which probably undergoes a conformational change favoring electron transport and substrate binding, during activation. Recently it has been demonstrated that $^{\bullet}O_2^-$ is controlled by multiple Rac GTPase activating proteins thus allowing selective regulation (12). NADPH oxidase catalyzes the production of $^{\bullet}O_2^-$ from molecular oxygen and NADPH (13). The superoxide anion ($^{\bullet}O_2^-$) is a mild oxidant that may further be converted into hydrogen peroxide (H_2O_2) spontaneously or by the action of superoxide dismutase (SOD). Furthermore, in a metal-catalyzed reaction (e.g. iron and copper) H_2O_2 can yield the highly reactive and instable hydroxyl radical ($OH^{\bullet}$) (14). Moreover, reaction between hydrogen peroxide and chloride results in the formation of hypochlorous acid (HOCl), which can further attack biomolecules both by oxidation and by clorination. This is usually catalyzed by myeloperoxidase (MPO), a member of the mammalian heme peroxidase superfamily, which is specific for macrophages and neutrophils that store the enzyme within the azurophilic granules. The MPO may be released inside the phagosomes during degranulation or even extracellularly by leakage either due to incomplete closure of the developing phagosome or due to stimuli by an antibody/complement-coated surface too large to be ingested, as in case of tumor cells (15). Due to the fact that MPO-derived ROS cause also host tissue injury by lipid peroxidation and protein modifications, MPO was thought to participate in chronic inflammatory diseases (16), while HOCl can enhance the immune response by oxidizing protein antigens. Beside proteins, lipids are also molecules that are readily affected by HOCl yielding chlorohydrins and lipid hydroperoxides (17). Also, HOCl can react with $^{\bullet}O_2^-$ leading to $OH^{\bullet}$ formation. Another enzyme released by neutrophils is extracellular superoxide dismutase (EC-SOD), the only extracellular enzyme with the ability to remove $^{\bullet}O_2^-$ (18). Although HOCl may oxidatively modify Met32 and induce intermolecular cross-link, under physiological relevant levels of HOCl, activity of EC-SOD is preserved (19).

Singlet oxygen ($^1O_2$) is another ROS produced by neutrophils. Singlet oxygen is formed in the reaction between H_2O_2 and an oxidized halogen and is likely to be responsible for some damage inflicted by neutrophils on their targets (1). Moreover, nitric oxide synthase (NOS) is another ROS producing enzyme in neutrophils yielding nitric oxide ($NO^{\bullet}$) from the conversion of the amino acid L-arginine to L-citrulline. Once formed, $NO^{\bullet}$ changes signaling pathways thereby exerting both pro- and anti-inflammatory effects (20). $NO^{\bullet}$ Superoxide anion may quench $NO^{\bullet}$ yielding the potentially cytotoxic compound peroxynitrite ($ONOO^-$). Peroxynitrite also functions in the physiological modification of signaling molecules by taking part in numerous reactions like oxidation, nitrosylation

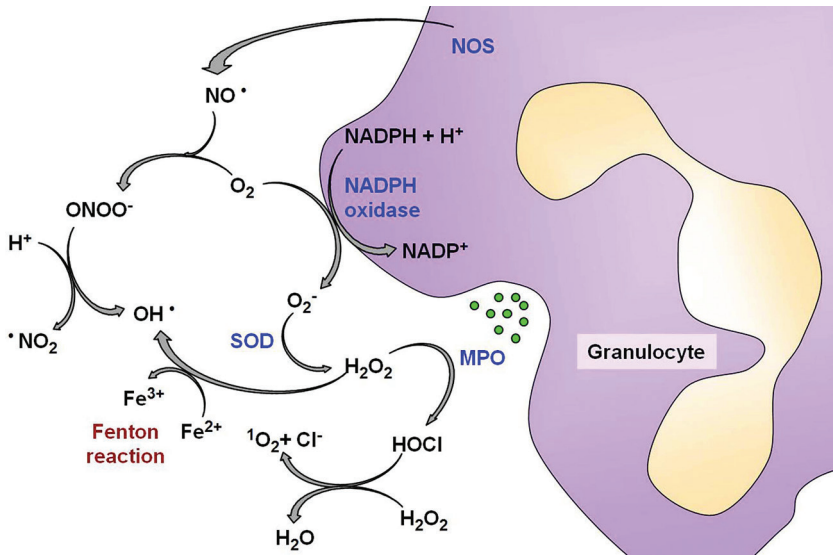

Figure 1. Oxidative burst of activated granulocytes. Membrane bound NADPH oxidase catalyzes extracellular production of $^{\bullet}O_2^-$ from O_2 and NADPH, which may further spontaneously or by the action of SOD dismutate to H_2O_2. Furthermore, H_2O_2 may take part in a metal (e.g. Fenton reaction) or MPO catalyzed reaction generating highly reactive $OH^{\bullet}$ or HOCl, respectively. Moreover, granulocyte NOS may yield extracellular $NO^{\bullet}$ that may take part in the reaction with $^{\bullet}O2^-$ leading to the generation of $ONOO^-$. Furthermore, $ONOO^-$ can undergo homolytic fission yielding $OH^{\bullet}$ and $^{\bullet}NO_2$ (for details please refer to the main text).

and lipid peroxidation (20). In biological systems $ONOO^-$ mainly reacts with carbon dioxide leading to formation of carbonate and nitrogen dioxide radicals ($^{\bullet}NO_2$) (21). Alternatively, ONOOH can undergo homolytic fission yielding $OH^{\bullet}$ and $^{\bullet}NO_2$. However, this reaction is slow in biological systems and therefore is a minor component of the *in vivo* reactivity of peroxynitrite (22). It was revealed recently that MPO is a potent endogenous scavenger of $ONOO^-$ acting as homeostatic enzyme that prevents uncontrolled decomposition of $ONOO^-$ and subsequent ROS formation (23).

Each neutrophil-derived ROS has a specific diffusion distance, which is related to the ROS lifetime and reactivity. For example, the highly reactive $OH^{\bullet}$ has a short diffusion range, while H_2O_2 has a relatively long diffusion range. Depending on their distance from a target the neutrophil-derived ROS may oxidize DNA, proteins, lipids and carbohydrates and thus mediate numerous redox mediated pathologies (Figure 2). Direct oxidation of amino acid residues of proteins may cause many post-translational modifications. Oxidation of other cellular components may lead to the formation of reactive intermediates that may also induce post-translational modification of proteins (24). Moreover, neutrophil-derived ROS are shown to modulate phagocytosis, cytokine secretion, gene expression, and apoptosis thus participating in autocrine and paracrine cell signaling that promotes inflammation (25).

3.1. ROS-induced lipid peroxidation

Another target of ROS are polyunsaturated fatty acids that are esterified in membrane or storage lipids and are subject to ROS-induced peroxidation and breakdown through non-enzymatic Hock cleavage resulting in the destruction of biomembranes (Figure 3) (26). Peroxidation of lipids results in the formation of unstable lipid hydroperoxides and finally, reactive aldehydes, having as consequence increased membrane permeability and fluidity, cytosol efflux and loss of membrane protein activities (26). Severe membrane dysfunction is usually associated with loss of viability. Transition metals also have a redox potential high enough to initiate lipid peroxidation. As expected, vast lipid peroxidation results in membrane disintegration and cell death, but it cannot be easily resolved whether it is a cause or a consequence of cell death (27). Final products of lipid peroxidation are reactive aldehydes such as 4-hydroxyalkenals and other similar α,β-unsaturated aldehydes, among which the most important are 4-hydroxynonenal (HNE), also considered as second messenger of ROS, malondialdehyde (MDA) and acrolein (28). The α,β-unsaturated aldehydes were shown to regulate signaling kinases through functional modification and thus regulate inflammation, apoptosis, and other signaling pathways (29, 30). A great number of these aldehydes have been isolated from biological samples, where they may promote and reinforce cell damage induced by oxidative stress (31). Lipid-derived aldehydes are more stable than ROS and can therefore diffuse across membranes and reach the targets distant from the initial site of oxidative injury (24). Such reactive aldehydes are subject to Michael addition reactions with the side chains of lysine, histidine, and cysteine residues. The common effect of protein carbonylation is enzyme inactivation i.e. loss of function (24) that is evidenced in numerous pathophysiological processes such as

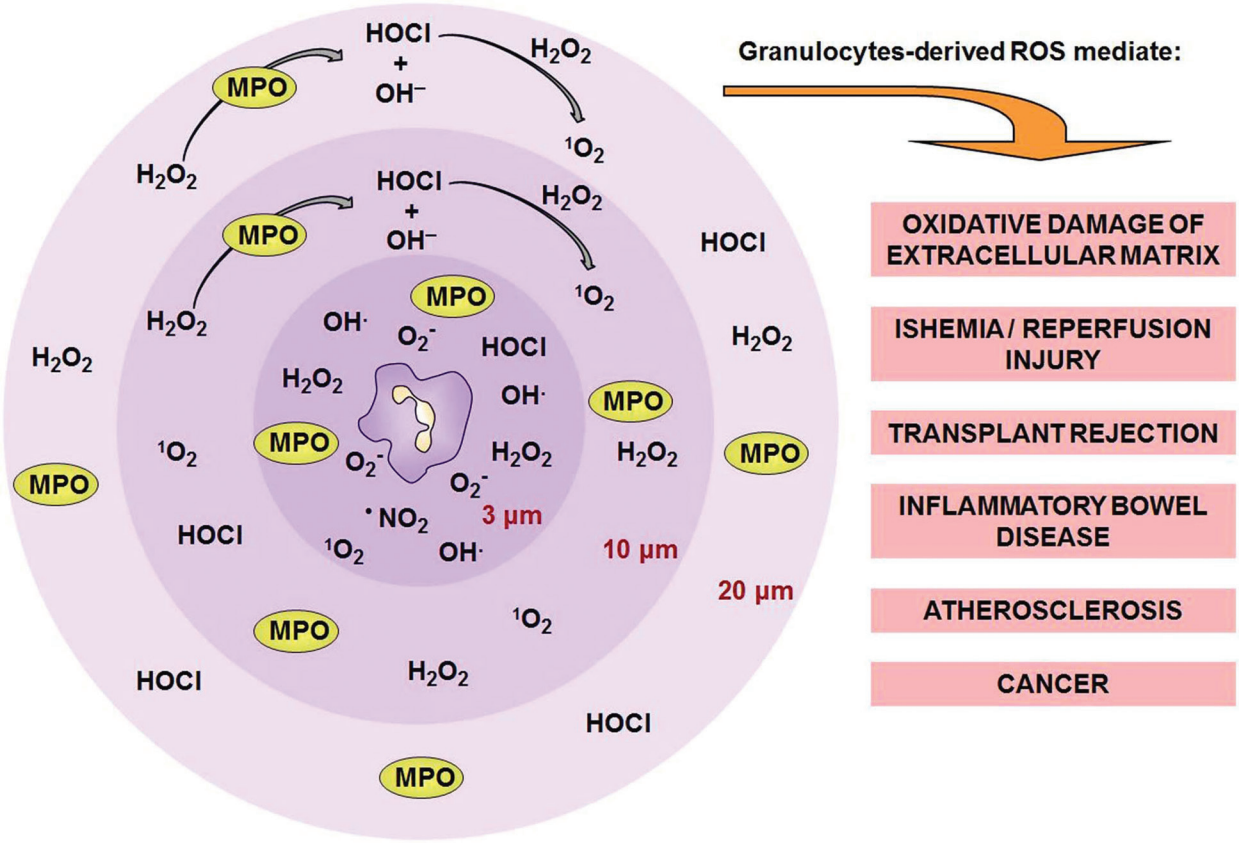

Figure 2. Granulocytes mediated extracellular redox pathophysiology. Activated granulocytes intensively produce ROS that have short (< 3 μM), medium (3 – 10 μm) or long (>10 μm) diffusion range. Excessive ROS induce oxidative damage of surrounding cells and tissue (e.g. extracellular matrix). Direct oxidative damage induced by activated granulocytes has been reported in numerous pathophysiologies like ischemia/reperfusion injury, transplant rejection, IBD, atherosclerosis and cancer development.

Na^+-K^+-ATPase inactivation in neurodegenerative disorders (32). However, protein carbonylation may also result in a gain of function (24), such as an indirect gain of function for Nrf2 (NF-E2-related factor 2) by up-regulation of its transcriptional activation (33). In response to oxidative stress, the transcription factor Nrf2 is considered as the central protein that interacts with the antioxidant response element sequence to activate gene transcription.

As described above modifications of metabolic and structural proteins play a significant role in protein dysfunctions, altering trafficking, processing of proteins, generating tissue damage and pathogenesis of numerous human diseases (34) (Figure 3).

4. ANTIOXIDATIVE DEFENSE SYSTEMS

Aerobic respiration and metabolism generate low levels of free ROS as byproducts even under physiological conditions. These ROS can be a part of normal cell signaling, but still, there is a need to keep the level of ROS in control by balancing between ROS generating and neutralizing processes. The physiologic homeostasis is maintained by optimal cellular and tissue-oxygenation status through oxygen-sensing mechanisms, signaling cascades, and transport processes (35). Disruption of this balance triggers protective redox signaling by induction of cellular protective mechanisms that re-establish the ROS/antioxidant balance. This is achieved through enzymatic and non-enzymatic antioxidative defense systems. Enzymatic antioxidative cascades are widely investigated and well described (Figure 4), and recently reviewed in details by Augustyniak A *et al* (36). In most cases of the oxidative stress, mutual interplay of the complex antioxidant network with cell signaling occurs, resulting in cell defense against ROS attacks on biomolecules and in cellular adaptation to stress. Neutrophils are the only cells primed to undergo severe oxidative stress physiologically ending in their decay that defends the other cells from aggressive stress, such as microbial or cancer invasion. Therefore, oxidative burst of neutrophils can result not only in the desirable defense, but also in the auto-destructive processes in various tissues. When the complex interplay between neutrophils and other cell types under such circumstances based

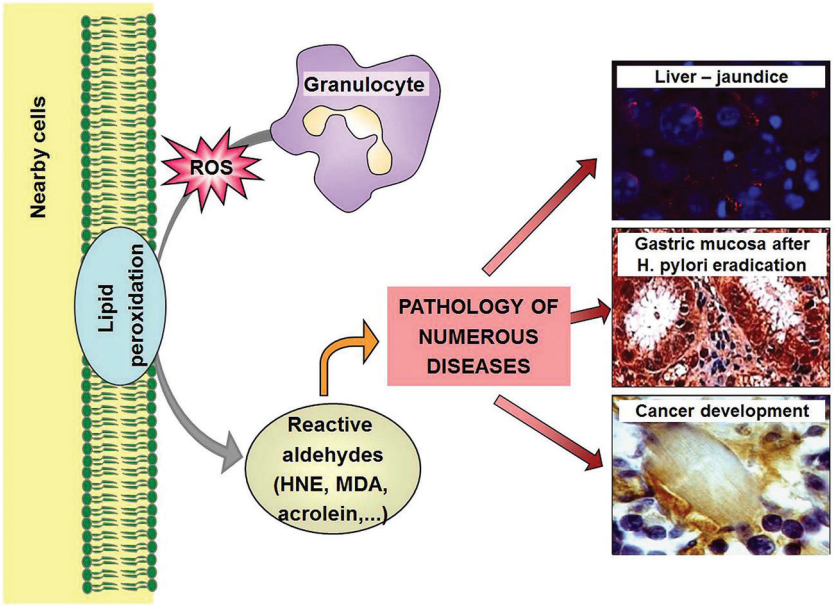

Figure 3. Secondary effect of granulocytes mediated extracellular redox signaling. ROS produced by granulocytes can induce lipid peroxidation of polyunsaturated fatty acids in the membranes of nearby cells yielding reactive aldehydes, among which are HNE, MDA and acrolein. Lipid derived reactive aldehydes are more stable than ROS and therefore can diffuse to targets distant from the initial site of ROS mediated injury. Lipid derived aldehydes are implicated in the pathology of numerous diseases, like jaundice, H. pylori eradication and cancer development. HNE-histidine adducts detected in liver sections of LEC rats (rat strain that accumulates copper in the liver and spontaneously develops hepatocellular carcinoma) at stage of slight jaundice (blue coloration= nuclei; red coloration=HNE-His positivity, confocal microscopy, magnification 400x). Five weeks after H. pylori eradication (magnification 200x), diffuse accumulation of HNE-histidine conjugates was observed, especially prominent in the nuclei of glandular cells. During cancer invasion of the sceletal muscle diffuse accumulation of HNE, especially prominent in the affected muscle is present, while cancer cells are mostly HNE-negative (magnification 400x).

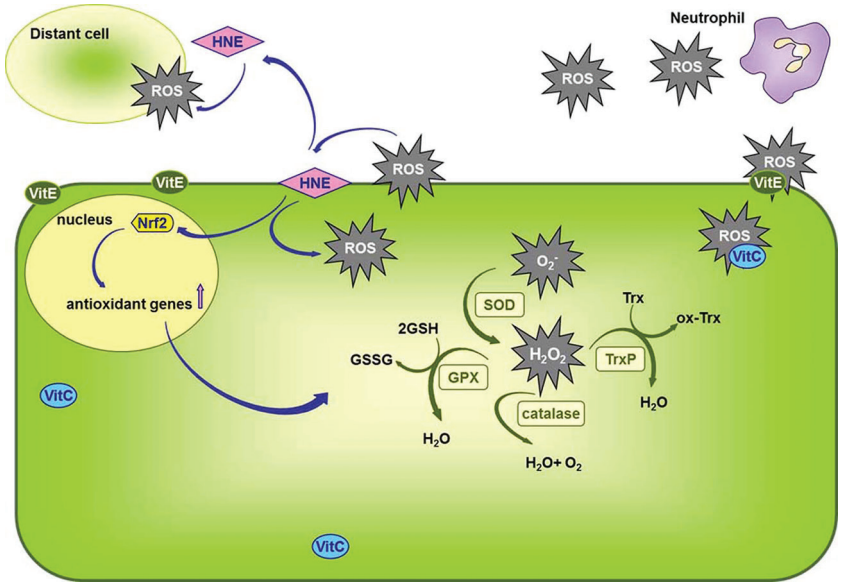

Figure 4. Cellular antioxidative defense mechanisms. Cells have developed several defense mechanisms against oxidative damage. Granulocytes produce ROS, and the first defense lines are vitamin E (VitE) in lipid membranes, and vitamin C (VitC) in aqueous compartments. Second, more specific defense lines are antioxidative enzymatic cascades. Here, superoxide anion is neutralized by superoxide dismutase (SOD), and the hydrogen peroxide is further neutralised by several reactions. Catalase decomposes H_2O_2 to water and molecular oxygen, glutathione peroxidase (GPX) oxidases glutathione (GSH), while thioredoxin peroxidase (TrxP) oxidases thioredoxin (Trx) to decompose hydrogen peroxide. Also, ROS cause lipid peroxidation, with HNE as end product. HNE can activate antioxidative enzymes by activating Nrf2, or can diffuse and contribute to further progression of ROS mediated damage.

on pro- and anti-oxidants, as well as cytokines and enzymatic systems would be well understood, it would be possible to improve diagnostics and therapies for the major, stress-dependent diseases of the modern society.

5. NEUTROPHIL INDUCED OXIDATIVE DAMAGE OF THE EXTRACELLULAR MATRIX

Majority of eukaryotic cells live in complex meshwork of collagens, glycoproteins, proteoglycans as well as growth factors, chemokines, cytokines and other components of interstitial fluid that jointly comprise the extracellular matrix (ECM). Due to the fact that neutrophils' oxidative burst occurs near the cell, ECM is susceptible to a greater oxidative stress than the targeted cell, resulting in structural changes of the matrix thus affecting cellular behavior. The changes in ECM structure have shown to have an important role in cell signaling, adhesion, migration and proliferation (37). Extracellular redox signaling mediated by neutrophils has an important role in ECM oxidative damage and can promote progression of inflammatory diseases (38). As described above, the activation process of neutrophils is accompanied by their oxidative burst, during which they extracellularly release $^{\bullet}O_2^-$ at the expense of cellular NADPH (13). The poorly reactive $^{\bullet}O_2^-$ may then dismutate to the more stable H_2O_2 yielding further radicals and oxidants. In addition, activated neutrophils may also extracellularly release MPO (as already mentioned) that yields HOCl, a strong oxidizing and chlorinating species. Also, the MPO is a highly cationic enzyme that eagerly binds to components of the ECM, like glycosaminoglycans and may result in their damage. The oxidative burst of neutrophils is thought to be involved in the loss of collagen and other matrix proteins at the inflammation site. Daumer *et al.* reported that HOCl affects type I and type II collagen resulting in the oxidation of amine groups, while for type II collagen it also acts by modification of pyridinoline cross-links, thus promoting the loss of matrix integrity (39). Low concentration of HOCl inhibits collagen gelation, while at high doses it induces collagen degradation (40). In addition, the interaction of neutrophil-derived ROS and collagen i.e. glycoxidation of collagen promotes further neutrophil migration and ROS production (37).

Fibronectin, an ECM glycoprotein, is also prone to HOCl induced oxidation resulting in either unfolding (at low HOCl concentrations) or in epitope destruction (higher HOCl concentrations) (37). Oxidative burst of neutrophils also affects glycosaminoglycans, like hyaluronan and chondroitin sulfate. Activated neutrophils induce depolymerization and degradation of glycosaminoglycans mainly by HOCl (37).

While we mentioned only the major ECM components that can be oxidatively damaged by activated neutrophils, it is certain that the majority of the extracellular as well as membrane proteins represent targets for neutrophils that can modify their structure and function. Thus, activated neutrophils are capable to oxidatively modify numerous molecular components of ECM (reviewed in (37)).

Modification of ECM by neutrophil-derived oxidants has been described in a number of inflammatory diseases, like arthritis and cardiovascular disease (i.e. atherosclerosis).

As we have described, oxidants mediate damage in inflammatory diseases but opposite to the common belief, antioxidant therapies did not have the desired effects (36). One of the reasons lies in the fact that there is a great diversity of oxidants with either hydrophilic or lipophilic properties. So, an antioxidant which acts in one cellular or tissue compartment does not have to have such efficiency in the other. Also, there is a problem of antioxidant administration and achievement of the optimal concentration in the right compartment and at the right time. Even more, one should have in mind that at low concentrations ROS are part of important redox signaling pathways (41). Although overproduction and accumulation of ROS is cytotoxic and damages macromolecules (DNA, proteins, sugars and lipids) (42), thereby causing degeneration of tissues, apoptosis, premature aging, mutagenicity and cancerogenesis, their removal could also be detrimental (reviewed in (37)).

6. NEUTROPHIL MEDIATED CHRONIC DISEASES

The involvement of neutrophils in the progression of chronic diseases is also well documented. The World Health Organization defines chronic diseases as diseases of long duration and generally slow progression. Chronic diseases, such as heart diseases, stroke, cancer, chronic respiratory diseases and diabetes are by far the leading causes of mortality in the world, representing 63% of all deaths. Therefore, we will in particular describe specificities of atherosclerosis and inflammatory bowel disease, as examples of diseases that have neutrophil activation at the basis of their pathology (with exception of cancer which will be described in a separate chapter due to the specific role of neutrophils in the pathology of cancer).

6.1. Atherosclerosis

Atherosclerosis is a primary cause of hearth diseases and cerebrovascular stroke and is thus the leading cause of death worldwide. A positive correlation between increased neutrophil counts and cardiovascular diseases was reported as well the activation and/or increased number of circulating neutrophils in numerous conditions associated with high risk of atherosclerosis. The retention of low density lipoprotein (LDL) in the ECM of the arterial intima is followed by the formation of atherosclerotic lesions and thus represents a crucial

factor in atherosclerosis. LDL is extremely susceptible to oxidative damage that may be induced by different cell types (e.g. endothelial cells, fibroblasts and neutrophils) and as such has been implicated in the pathogenesis of atherosclerosis (43). Peripheral blood neutrophils oxidize LDL by superoxide reactions and by HOCl produced by MPO in the arterial intima (17). Furthermore, HOCl modifies LDL by oxidizing thiol and methionyl residues as well as by the formation of chloramines. Moreover, both the lipid and protein moiety of LDL could be efficiently modified by the MPO-H_2O_2-halide system (44). During oxidation of LDL, the peroxidation of the unsaturated fatty acids of LDL lipids may occur yielding the formation of reactive aldehydes. Not only ROS, but MPO as well was shown to induce lipid peroxidation of LDL (45). Therefore, the importance of lipid peroxidation and its aldehydes will be described below. In addition, NADPH oxidase and MPO released by activated neutrophil can impair the endothelial function by catalyzing the reactions that consume vascular NO (46). Also, the excessive release of $^{\bullet}O_2^-$ by activated neutrophils at the blood-tissue interface may facilitate chronic minimal injury to the endothelium and thus contribute to the initiation of atherosclerosis (47,48). Taking together all the evidence, neutrophils mediate inflammation thus contributing to the plaque development and vulnerability of the affected vessels. Zernecke *et al* have provided evidence that neutrophils crucially contribute to atherogenesis. They also identified a protective role for Cxcr4/Cxcl12 through controlling neutrophil homeostasis and their recruitment to plaques (49). It was also demonstrated that Cxcr4 blockage leads to progressive plaque expansion and contributes to pathogenesis of atherosclerosis (50).

6.2. Inflammatory bowel disease

Inflammatory bowel diseases (IBD) comprise chronic idiopathic inflammatory disorders of the digestive tract, especially intestine and colon, characterized by rectal bleeding, severe diarrhea, abdominal pain, fever and weight loss. Biopsies reveal large number of leukocytes such as neutrophils in the intestine and/or colon. The inflammatory infiltrate is accompanied by extensive mucosal and/or transmural injury including edema, loss of goblet cells, decreased mucous production, crypt cell hyperplasia, erosions and ulcerations (51). Although still under debate, recent experimental studies suggest that dysregulation of the immune response to components of normal gut flora is amenable for chronic gut inflammation (52). The mechanisms by which the dysregulated immune response initiates and enhances intestine injury are not clarified yet. Still, any inflammation associated with enhanced production of reactive oxygen and nitrogen species could destroy affected tissues, including intestinal tissues (53). Since neutrophils are the primary source of ROS during inflammation, this review will highlight some of the basic concepts related to role of neutrophils in the pathology of IBD.

The intestinal mucosal interstitium is continuously exposed to large amounts of antigens, which can be either endogenous or exogenous. The mucosal immune system has developed efficient mechanisms to distinguish pathogenic, non-pathogenic and normal gut flora. Effector functions are regulated by a specific subset of T-helper cells (Th). These cells secrete cytokines such as tumor necrosis factor α (TNF α), IL-1b, IL-6, IL-8, and IL-12 which result in activation and recruitment of neutrophils to engulf and destroy invading microorganisms. Failure to regulate this protective response is thought to be a key event in the pathogenesis of IBD (53).

In addition, the inability to regulate tissue macrophage activation together with activation of neutrophils has as a consequence augmentation of ROS production. The production of ROS is usually attenuated by cellular antioxidant protection. Namely, cells have enzymatic (SOD, catalase, glutathione peroxidase) and non enzymatic (L-ascorbic acid, α-tocopherol, glutathione and other thiols) antioxidative defense systems (reviewed in chapter 8). When ROS are overproduced, the cell/tissue antioxidant defense is unable to limit oxidative damage, thereby causing progressive injury. During the active episodes of IBD uncontrolled production of ROS overwhelms the antioxidative protection of intestinal tissues and causes significant oxidative stress (53). As mentioned before the NADPH oxidase is a key enzyme in the oxidative burst of neutrophils and is therefore also associated with increased oxidative burst of neutrophils in the intestine of the IBD patients (53). While oxidative burst yields $^{\bullet}O_2^-$ and H_2O_2 that are not particularly deleterious, in the present of ferrous iron the highly reactive and deleterious hydroxyl radical is yielded (the Fenton reaction). Although neutrophils do not produce high amounts of the hydroxyl radical, $^{\bullet}O_2^-$ can cause release of redox active iron from ferritin, the storage protein of iron. Further, bleeding is another source of redox-active iron. Taking all these events together, a vicious circle is formed and free radical damage propagates. Yet, these are not the only ROS formed by neutrophils. HOCl formed by neutrophils also has an essential role in IBD pathology. HOCl oxidizes proteins and interacts with $^{\bullet}O_2^-$ through non-Fenton reaction producing the hydroxyl radical (54). Last, but not least, MPO or eosinophil peroxidase may catalyze oxidation of other halides and pseudohalides, such as bromide or thiocyanante to yield hypobromous or hypothiocyanous acid, respectively. These oxidants may also have a role in the pathology of IBD (53).

7. ISCHEMIA REPERFUSION AND TRANSPLANTATION INJURY

Ischemic injury occurs when blood flow to the organ or tissue is cut off, while reperfusion is the restoration of the blood flow. Myocardial infarction, stroke, and other thrombotic events are only some examples of ischemic

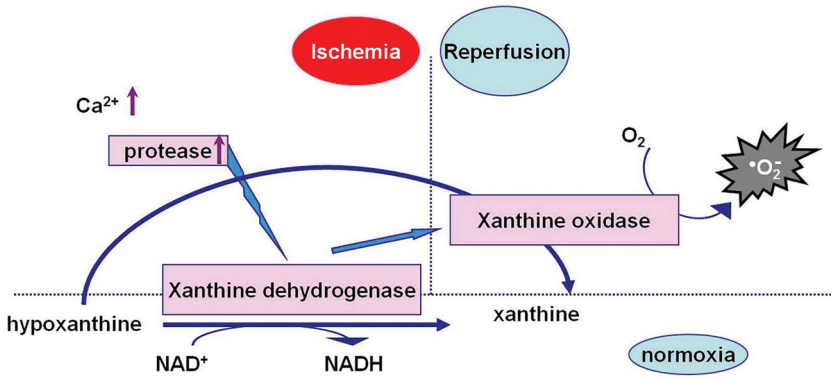

Figure 5. Ischemia reperfusion injury. During ischemia calcium ions are released to the cytosol, where activation of proteases occurs. Once activated a protease converts xanthine dehydrogenase to xanthine oxidase. At the same time, cell converts ATP to AMP which is further degraded to hypoxanthine. After reperfusion, hypoxanthine is converted to xanthine by xanthine oxidase, which uses O_2 instead of NAD^+, with $^{\bullet}O_2^-$ as byproduct.

injury. Due to oxygen deprivation many metabolic changes occur during ischemia. One of them is calcium ion influx. Calcium ions thus activate the protease that converts xanthine dehydrogenase to xanthine oxidase (Figure 5) (55). At the same time, adenosine triphosphate (ATP) is catabolized to adenosine monophosphate (AMP) and finally to hypoxanthine. These events are in favor of free radical production when blood flow is re-established. In normal, aerobic conditions, xanthine dehydrogenase uses NAD^+ to convert hypoxanthine to xanthine. On the other hand, xanthine oxidase oxidizes hypoxanthine to xanthine, and further oxidizes xanthine to produce uric acid, $^{\bullet}O_2^-$, and H_2O_2. The net result of reperfusion is the increase in ROS production, which further leads to oxidative stress and lipid peroxidation (Figure 5).

Tissue undergoing reperfusion is injured and secretes different chemoattractants. Complement fragments C5a and $C5a_{des\ arg}$, and cytokines such as TNFα, IL-1, IL-6, IL-8, NAP-1 (Neutrophil Activating Peptide-1), PAF (Platelet Activating Factor) are some of the factors that attracts neutrophils and stimulate neutrophil events in ischemic-reperfused tissue (56). In addition, the lipid peroxidation product HNE increases ROS formation and acts also as chemoattractant for neutrophils. Also, some of the mentioned cytokines released by degranulation of mast cells, together with other factors released by mast cells such as histamine, serotonine, and PAF, participate in the inflammatory response to ischemia reperfusion injury. Further, reperfusion stimulates shape changes of neutrophils necessary for migration, chemokinesis and increased adherence to endothelium as was shown by sampling lymph during reperfusion (56). Finally, neutrophils contribute to cell necrosis directly or indirectly by several pathways: 1) oxidative burst and production of ROS causing membrane protein oxidation and membrane disruption; 2) release of proteases such as elastase and collagenase; 3) embolisations of microvessels causing secondary ischemia; 4) damage of vascular endothelium causing upregulation of adhesion molecules further enhancing neutrophil adhesion and migration; 5) release of mediators that amplify further recruitment of neutrophils to reperfused tissue which induce vasoconstriction and platelet activation; 6) promotion of interactions with platelets that potentiates ischemic-reperfusion injury; 7) extension of the degree of apoptosis during later phase of reperfusion (56). A growing body of evidence supports the link between early neutrophil recruitment and size of tissue damage, thereby indicating their role in ischemic-reperfusion diseases such as infarction but also in transplantation injury. While transplant rejection mediated by T-lymphocytes due incompatibilities of the major histocompatibility complexes is well known (57), relatively little attention is put on role innate immunity mediated by neutrophils in the same pathophysiological process. Hence, the relevance of the innate immunity in transplantation biomedicine should be more intensively studied, while studies carried out so far did not give unambiguous results.

The etiology of primary graft rejection is multifactorial, including cold ischemia time and ischemia/reperfusion injury being one of the most important contributors (58). As mentioned, it is inevitable that during organ transplantation tissue undergoes ischemia/reperfusion injury. The mechanisms by which neutrophils contribute to transplant rejection are numerous. Hence, beside oxidative burst and ROS production (59) their role in cytokine secretion as well as being the antigen presenting cells to T lymphocytes became evident (60). These findings might open a novel approach to the transplantation biomedicine, which might eventually also improve our basic knowledge about the functional activities of the neutrophils, too.

8. CARCINOGENEIS AND SPONTANEOUS REGRESSION OF CANCER

ROS have very important roles in tumor biology (61) and are thought to have both a tumor

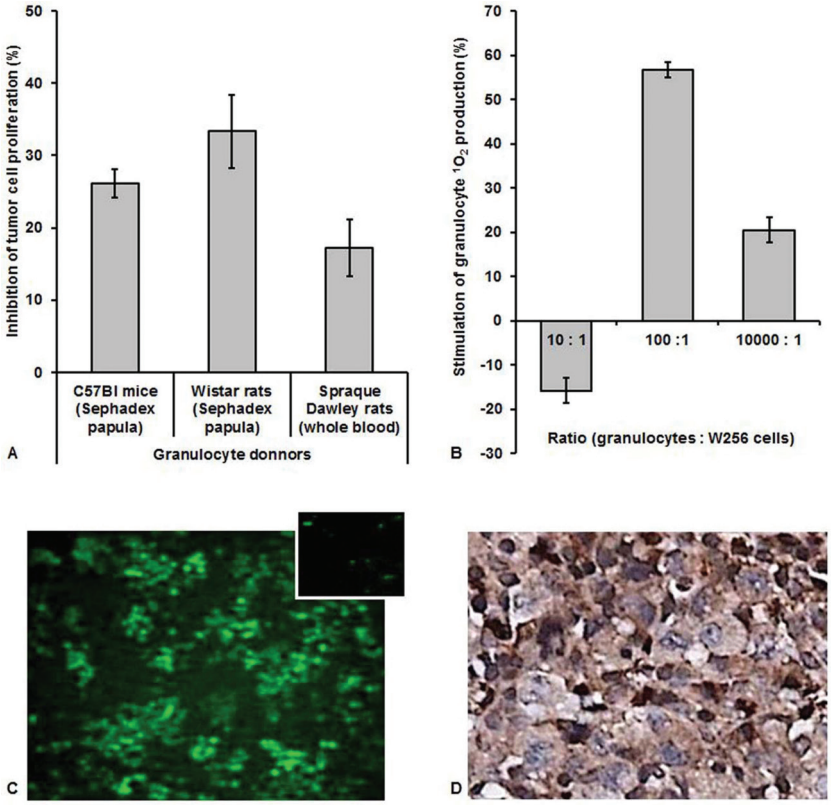

Figure 6. Granulocytes challenged with tumor cells. A. Granulocytes, isolated from subcutaneously formed Sephadex papula of healthy C57Bl mice (65) and Wistar rats (8) or isolated from whole blood of healthy Sprague Dawley rats (66), inhibit tumor cell proliferation (median ± SE). B. Functional activity of granulocytes, measured by luminol-enhanced chemiluminescence (8), is decreased at granulocyte:tumor cell ratio 10:1, while it is increased when granulocytes are present at a higher ratio such as 100:1 and 10000:1 (median ± SE). C. W256 tumor cells stimulate granulocyte intracellular ROS production, as measured by 2,7-dichlorodihydrofluorescein diacetate fluorescence assay (magnification 100x). In the small picture are solely granulocytes. (Jaganjac *et al*. Submitted, unpublished observations). D. Excessive infiltration of granulocytes at the site of W256 transplantation in Sprague Dawley rats observed on the 2nd day is accompanied by strong MPO presence (magnification 400x) (Jaganjac *et al* unpublished results).

promoting and tumor suppressing effects. 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 (62). However, a number of researchers have reported cytotoxicity of neutrophils against tumor cells *in vitro* (63, 64) and *in vivo* (8,65-67). Therefore this section will be in particular focused on neutrophil mediated tumor cell cytotoxicity, although one should keep in mind that, if neutrophils do not cause cell death, secreted ROS can also promote tumor growth as already mentioned.

Recently, we have reported that spontaneous regression or complete resistance to cancer cells is mediated by rapid infiltration of neutrophils, mostly as a consequence of innate immune response (66). In addition, the administration of neutrophils at the site of solid tumors can lead to tumor regression or can slow down tumor growth and extend the overall survival of animals (67). As the size of tumor cells is several times bigger than that of microorganisms, the ingestion of tumor cells by neutrophils is not likely to occur. However, it seems that the mechanisms essential for killing microorganisms are also important in neutrophil mediated tumor cell cytotoxicity.

8.1. Unspecific lysis of tumor cells

It was demonstrated that activated neutrophils cause unspecific lysis of tumor cells mediated by ROS, hence ROS have been identified as effector molecules of the oxygen-dependent killing of cancer cells by neutrophils (68). We revealed that oxidative burst of neutrophils has an important role in tumor progression (8,65) or regression (66, 67) describing its cytotoxic effect for melanoma B16F10, Ehrlich ascytic tumor and for Walker 256 carcinoma (W256) (Figures 6 and 7). Oxidative burst of neutrophils is already pronounced in tumor-bearing animals in the earliest stage of tumor development, while further tumor progression is associated with a constant increase in the oxidative burst of neutrophils. In case of tumor regression the oxidative burst of neutrophils decreases to normal

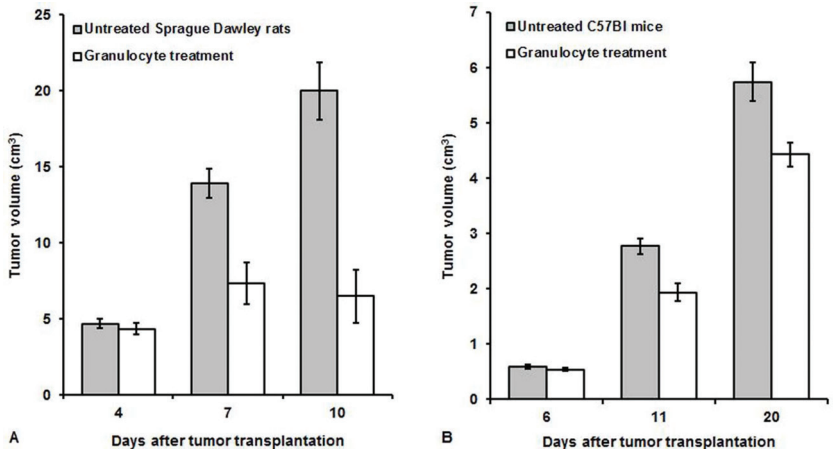

Figure 7. The administration of granulocytes at the site of solid tumors. A. Spontaneous W256 tumor regression in Sprague Dawley rats was abolished by distraction of functional granulocytes from the blood. The restoration of the functional granulocytes obtained by granulocyte administration on the 4th day at the site of tumor led to tumor regression (median ± SE) (67). B. The administration of granulocytes on the 6th day at the site of Ehrlich ascites tumor grown in BALBc mice resulted in slower tumor growth thus extending the overall survival of animals (median ± SE) (67).

values accompanying the tumor disappearance (66). Therefore, one may assume that an elevated functional activity of neutrophils may result in tumor regression while continuous growth of tumor is associated with gradual increment in neutrophil oxidative burst that might even promote the tumor progression. Although this seems paradoxical, it is not. Namely, as already mentioned excessive ROS can induce lipid peroxidation that acts as a double-edged sword in carcinogenesis exhibiting either a pro- or anti-tumor effect.

The most specific and the most important neutrophil enzyme MPO is necessary for antitumor activity of neutrophils. The MPO generated HOCl appears to have a crucial role in neutrophil mediated malignant destruction. HOCl has been shown to enhance the immunogenicity of tumor cells thus inducing rapid necrosis of tumor cells (69). Thus, HOCl is thought to play a crucial role during MPO-based attack on tumor cells.

Both prokaryotic and eukaryotic cells respond to this toxicity by protection mechanisms coordinately inducing a series of genes encoding detoxifying and antioxidative stress enzymes/proteins that provide the necessary protection against oxidative and electrophilic stress as described in chapter 8 (70). Although cancer cells have less potent antioxidant capacities than the non malignant cells, the transformed cells are often even more resistant to oxidative stress due to the differences in lipid metabolism.

It has been suggested that one of the mechanisms by which oxidative burst of neutrophils may lead to tumor destruction could be by influencing the Nrf2 signaling pathway (71). ROS can often function in multiple places (i.e. upstream or downstream) within a given pathway, like in the NF-κB pathway. ROS can stimulate the NF-κB pathway in the cytoplasm, but inhibit NF-κB pathway in the nucleus (72). Thus, ROS produced by oxidative burst of neutrophils may influence the NF-κB signaling pathway by repressing the Nrf2-ARE pathway and thus lead to malignant destruction (71).

Several recent breakthroughs revealed that oxidative burst of neutrophils triggers a unique cell death pathway where mixing of chromatin and granular antibacterial protein occurs in neutrophils, consequently being followed by the rupture of the neutrophil plasma membrane and ending in neutrophil extracellular trap (NET) formation (73). NETs arise from neutrophils that have activated a cell death program called “NET cell death”, or “NETosis”. NETs are composed of mitochondrial DNA, histones, granule enzymes, and antimicrobial proteins that are released by the neutrophils in parallel with extrusion of nuclear material (74, 75). Uric acid, a product of purine metabolism and $^1O_2$ scavenger, was recently shown to directly induce NET formation, however the underlying mechanism is still not clear (76). It was reported that DNA complexes present in NET capture and extracellularly kill both bacteria and fungi (77). Mitochondrial DNA can further activate neutrophils via TLR9 signaling (78). NETs are also implicated in vascular injury (79) therefore we could assume that coordinated action between intercellular ROS signaling and NET (80) could have an important role in neutrophil mediated tumor cell destruction.

8.2. Neutrophils and tumor cells – intercellular redox signaling

The term intercellular ROS signaling was suggested by Bauer and his colleagues. They described four ROS intercellular signaling pathways:

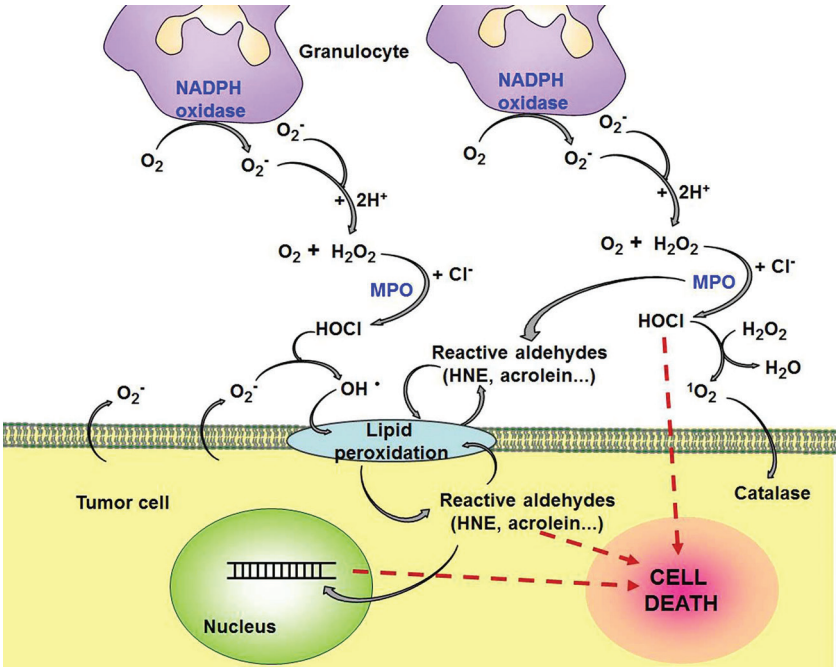

Figure 8. Granulocytes and tumor cell – intercellular redox signaling. The presented HOCl signaling pathway depends on granulocyte and tumor cell $^{\bullet}O_2^-$ generation. NADPH oxidase generates $^{\bullet}O_2^-$ that further dismutates to H_2O_2. MPO further catalyzes H_2O_2 conversion into HOCl. HOCl can react with tumor cell produced $^{\bullet}O_2^-$ yielding highly reactive OH• that induces lipid peroxidation. Lipid peroxidation derived reactive aldehydes further contribute to tumor cell lipid peroxidation and contribute to malignant destruction (for details please refer to the main text).

NO/peroxynitrite, nitryl chloride, metal catalyzed and HOCl signaling pathway (81). The intercellular induction of apoptosis is mainly accomplished by the HOCl signaling pathway. This pathway depends on the $^{\bullet}O_2^-$ generation from both transformed cells and neutrophils (Figure 8). Neutrophils employ NADPH oxidase to generate $^{\bullet}O_2^-$ that further dismutates to H_2O_2. By the action of MPO, H_2O_2 is further converted into reactive HOCl. At low levels HOCl does not affect cells directly; however, at high concentrations it exhibits its cytotoxic effect either directly or indirectly by modifying biomolecules. Also as described above, the interaction of HOCl with $^{\bullet}O_2^-$ yields highly the reactive $OH^{\bullet}$ that can trigger apoptosis through induction of lipid peroxidation. Moreover, neutrophils employ the MPO-hydrogen peroxide-chloride system to convert free hydroxy-amino acids into highly reactive α,β-unsaturated aldehydes, like HNE and acrolein (82). At the site of tumor implantation we have observed rapid infiltration of neutrophils (66) and pronounced MPO presence accompanied by acrolein formation during tumor regression (Jaganjac *et al.* unpublished observations). Acrolein can induce ROS formation that was suggested to be responsible for the induction of lipid peroxidation (83). Thus, end products of lipid peroxidation, such as HNE and acrolein, further contribute to the progression of neutrophil mediated oxidative damage of tumor cells. Apoptosis induction in transformed cells depends exclusively on extracellular ROS during the first 20 hours of malignant transformation, indicating a crucial role of ROS signaling in early carcinogenesis (84). Interactive ROS signaling was also suggested to be used by natural host antitumor systems during the induction of selective apoptosis in transformed cells (81). Since early carcinogenesis is also associated with the neutrophil response to malignant cells, we may assume that neutrophils contribute in such a specific anticancer defense.

Eventually, both ROS and reactive aldehydes might react with DNA, lipids and proteins of the malignant cell resulting in the disruption of its transformed functional integrity consequently leading to destruction of cancer. Such a desirable, specific anti-cancer effects were recently revealed for HNE acting as dose-dependent inhibitor of cancer-specific catalase defense against ROS, which may also explain the crucial importance of extracellular redox reactions involving HNE and matrix remodeling in cancer regression (85,86).

9. CONCLUSIONS

Neutrophil-mediated extracellular redox signaling may have either beneficial or detrimental role in various pathophysiological processes. The manner in which ROS will act depends on the cell and tissue types involved, the (extra)cellular location of ROS production and the dynamic changes of individual ROS levels. Neutrophil-derived ROS primarily exhibit their effects by

direct attack on structures of nearby cells and surrounding tissue. Also, oxidative burst of neutrophils can defend the other cells from aggressive stress, but also can take part in the auto-destructive processes of various tissues. However, neutrophils do not only affect the nearby cells but also distant ones. Namely, neutrophils induce lipid peroxidation resulting in reactive aldehydes that may reach targets far from the initial site of ROS attack. Therefore, by this secondary effect of neutrophils, the influence of their oxidative burst radically increases to yet inconceivable proportions. Furthermore, depending upon the concentration utilized, the lipid-derived aldehydes may also have either beneficial or detrimental effects on cells. Consequently, when studying the oxidative burst of neutrophils, it is very important also to take into account its secondary effects, mainly accomplished by reactive aldehydes, especially 4-HNE and acrolein. Taken together, it is important to maintain homeostasis in the cell as well as in the organism, to keep the immune system on the "safe side", acting against pathogens and not against itself. Indeed, without the knowledge of the principles of hormesis, especially involving reactive aldehydes like HNE, it is not possible to get a full picture about beneficial or detrimental effects of neutrophils and other phagocytes (macrophages) not only in cancer but also in other stress-associated diseases (87-89).

10. ACKNOWLEDGEMENTS

The study was supported by COST B35, CM1001 and EU-ROS Actions of the European Union.

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Key Words: Neutrophils, Oxidative Burst, Chronic Diseases, Ischemia Reperfusion, Transplantation Injury, Cancer, Review

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