Neutrophils and respiratory tract DNA damage and mutagenesis: a review

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Inflammation has been recognized as an important factor in cancer development. For the lung, experimental studies with rats, as well as molecular epidemiological studies in humans, have provided evidence that the influx of neutrophils into the airways may be an important process linking inflammation with carcinogenesis. Currently it is believed that the genotoxic capacity of neutrophils is a crucial aetiological factor in this carcinogenic response. In the present review we discuss two major pathways of neutrophil-induced genotoxicity: (i) induction of oxidative DNA damage through release of reactive oxygen species (ROS) and (ii) myeloperoxidase (MPO)-related metabolic activation of chemical carcinogens. So far, direct evidence for a role of neutrophils in pulmonary genotoxicity has largely been derived from in vitro studies using co-cultures of activated neutrophils and target cells. Current evidence from in vivo studies is primarily indirect and additional animal studies are needed to substantiate causality. A further challenge will be to extrapolate results from such studies to humans. Taken together, this will provide a better insight into the role of neutrophils in pulmonary carcinogenicity and may, hence, lead to novel approaches for cancer prevention strategies.

Introduction

The association between chronic inflammation and the development of cancer has been recognized for a long time (1, 2). In fact, as early as the late 1800s, Rudolf Virchow described the appearance of tumours developed at sites of chronic irritation (3). Currently, there is a large number of clinical data confirming these early observations. Colon carcinomas are, for example, closely associated with chronic inflammatory bowel diseases, including colitis ulcerosis (4). Similarly, patients with chronic infections, such as osteomyelitis or decubitus ulcers, can develop very aggressive carcinomas (5) and urinary bladder cancer is frequently found in patients who suffer from urinary tract infection (6). Also in the lung, chronic inflammatory diseases, such as sarcoidosis, fibrosis and chronic obstructive pulmonary disease (COPD), have been associated with a higher risk of cancer development (2, 7–10). In addition, other studies demonstrated that human lung cancer risk can be modified by polymorphisms in pro-inflammatory genes such as IL-1β, TNF-α (11, 12). The strongest support for the involvement of chronic inflammation in carcinogenesis, however, is possibly derived from studies showing that long-term users of anti-inflammatory drugs may be at a reduced risk of cancer development (13).

Within the lung, many sources of inflammation may be effective in accelerating carcinogenesis, including inflammatory reactions caused by air pollution particles (e.g. cigarette smoke, diesel, quartz) [for reviews see refs (14 and 15)]. Inflammatory processes in the lung are characterized by the influx of neutrophils into the airways, and it is generally accepted that these cells are crucially involved in the pathogenesis of various chronic pulmonary diseases. The pulmonary vasculature represents the largest reservoir of circulating polymorphonuclear neutrophils (PMN) in the human body. The concentration of neutrophils within the pulmonary capillary blood is 35–100 times greater than within the large vessels of the systemic circulation, thereby providing a pool of rapidly recruitable neutrophils (16). Neutrophils are a major source of oxidants in the inflamed lungs, and the constant release of reactive oxygen species (ROS) by these cells provides a plausible mechanism by which inflammatory processes and pulmonary carcinogenesis might be related: ROS cause genetic alterations in the lung epithelium, which may promote cancer development (17). In this paper we provide a review of the DNA damaging and mutagenic capacity of neutrophils as well as a discussion of the implications of these processes for pulmonary carcinogenesis. We will specifically focus on the lung, as the concept of neutrophil-infiltration in relation to tumour development is largely derived from studies investigating the pulmonary pathogenicity of inhaled particles [for reviews see refs (14, 15 and 18)]. This review will highlight two distinct pathways of neutrophil-induced genotoxicity: (i) oxidative DNA disturbances caused by neutrophil-derived ROS and (ii) myeloperoxidase (MPO)-mediated metabolic activation of inhaled chemical carcinogens. It should be realized that induction of DNA damage and mutagenesis alone does not cause cancer and that neutrophils, for instance by generating a pro-oxidant environment, as well as by releasing defensins (19), proteases or growth factors may have an impact on a variety of other neoplastic processes including cell proliferation, metastasis and angiogenesis. However, these topics are beyond the scope of this paper and are discussed in several excellent reviews [see refs 1, 2, 17 and others].

Pulmonary neutrophils: friend or foe?

Inflammation is a normal response of tissues to ‘inactivate’ invading microbial, chemical or physical agents. A key component in the inflammatory reaction is the transport and accumulation of phagocytes into the injured tissue. The role of

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neutrophils in host defence was first discovered in the late 1800s by Elie Metchnikoff, who observed that ‘wandering’ cells crowded around sharp thorns, which he had pierced into transparent starfish larvae (20). In the lung, the recruitment of neutrophils upon inhalation of inflammogenic agents largely depends on an elaborate network of cytokines (21). In this process, resident alveolar macrophages have a pivotal role. In normal conditions, alveolar macrophages clear most of the invading micro-organisms or particles. However, when macrophages are overwhelmed by the intrinsic inflammogenic activity of the pathogens, they will be activated to release a wealth of cytokines and chemokines, eliciting a rapid inflammatory reaction. In general, neutrophil recruitment is characterized by three subsequent steps: adherence, extravasation and migration (22,23). The adherence of PMN to the vascular endothelium is mediated by specific adhesion molecules (22). Subsequent processes of pulmonary neutrophil recruitment (extravasation and migration) are controlled by chemokines including IL-8 (24–26). For a more detailed description of the processes involved in neutrophil trafficking into the airways, readers are referred to other reviews [see refs (16, 22 and 27–29)]. Once migrated into the lung, the PMN will be activated to release a myriad of products dedicated to kill invading pathogens. Currently, >50 neutrophil-derived toxins have been identified including proteolytic enzymes and bactericidal proteins [for extensive reviews see refs (22, 27 and 30)]. A hallmark of neutrophil activation, however, is their respiratory burst. This is characterized by an increased consumption of oxygen that is used to generate large amounts of ROS. This process ultimately leads to the conversion of H2O2 into hypochlorous acid (HOCl) by the neutrophilic enzyme MPO. HOCl is a very strong oxidant, which plays a crucial role in actual killing of inhaled pathogens by migrated neutrophils (31).

In contrast to their protective role in innate immunity, PMN are also implicated in the pathogenesis of a variety of acute or chronic inflammatory pulmonary diseases including chronic obstructive pulmonary disease (32,33), idiopathic pulmonary fibrosis (34) and the adult respiratory distress syndrome (35). In these diseases neutrophils are associated with several pathogenic processes. For instance, PMN-derived oxidants and proteolytic compounds are implicated in a great variety of injurious effects, including lung matrix degradation, killing of pulmonary epithelial and vascular endothelial cells, and inducing detachment of these cells from their matrix support (36,37). Furthermore, oxidants released by neutrophils might have a role in deregulation of the airway smooth muscle function, disturbing its contractile response (38). Finally, neutrophil-derived ROS cause oxidative DNA damage that may provoke mutagenesis in pulmonary target cells.

**Neutrophils as a source of ROS**

The most significant and important ROS-generating system in the lung is constituted by the pool of inflammatory neutrophils. In course of their defence activities, they produce a vast amount of oxidants. The whole spectrum of oxidants generated by neutrophils is more or less due to the action of four different enzymes, catalysing different reactions (summarized in Table I). NADPH-oxidase is the enzyme by which the oxidant generation is initiated. It is a membrane-bound enzyme, which is dormant in resting cells but comes into action when the cell is activated by phagocytosis of invading micro-organisms or particles. NADPH-oxidase is composed of a number of subunits, which are distributed in the cytosol and the membranes of intracellular vesicles and organelles. Upon activation, the cytosolic subunits migrate to the membranes where they bind to membrane-bound subunits, assembling the active oxidase. The intracellular organelles then fuse with the plasma membrane resulting in the release of O2− into the extra-cellular environment or into phagocytic vesicles (39). This O2− is a substrate for the enzyme superoxide dismutase (SOD), which catalyses the formation of H2O2 from O2− (40). H2O2 is relatively stable and is known for its capacity to diffuse and to cross cellular membranes. As such, this provides a phagocyte the possibility to ‘act at a distance’. In neutrophils, however, most of the hydrogen peroxide (up to 70%) is consumed by the enzyme MPO (41). MPO is the most abundant protein in neutrophils and catalyses the conversion of H2O2 into HOCl (31). In addition, neutrophils are also able to produce reactive nitrogen species, which is facilitated by inducible nitric oxide synthase (iNOS), catalysing the production of NO• from L-arginine, oxygen and NADPH (42). However, the ability of neutrophils to yield NO• is much less compared with macrophages (43,44), and studies on the activity of iNOS in isolated blood neutrophils have yielded contradictory results (45,46). The enzymes as listed in Table I give rise to the four oxidants initially generated by activated neutrophils, O2•−, H2O2, HOCl, NO•. Because of their reactivity, these products will constantly interact with each other, causing the formation of a myriad of oxidants (see Figure 1) among which the hydroxyl radical is recognized as being the most DNA-reactive compound by far (47,48).

**Table I. Enzymes and reactions involved in oxidant generation by neutrophils**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Reaction</th>
<th>Product</th>
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<tbody>
<tr>
<td>NADPH oxidase</td>
<td>2O2− + NADPH → 2O2•− + NADP+ + H+</td>
<td>O2•−</td>
</tr>
<tr>
<td>SOD</td>
<td>2O2•− + 2H+ → O2− + H2O2</td>
<td>H2O2</td>
</tr>
<tr>
<td>MPO</td>
<td>Cl− + H2O2 → HOCl + OH−</td>
<td>HOCl</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>L-arg + O2− + NADPH → NO• +</td>
<td>NO•</td>
</tr>
<tr>
<td>synthase</td>
<td>L-citrulline + NADPH</td>
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</table>

**Oxidant-induced genotoxicity by neutrophils**

Genotoxic processes may lead to irreversible changes in the structure of the genetic material of cells. This effect is considered as an important process in cancer development. The term 'genotoxic' has been used for the first time in 1973 by Hermann Druckrey during a conference on ‘Evaluation of genetic risks of environmental chemicals’ in Sweden. He stated: ‘In order to describe the components of chemical interaction with genetic material, the term genotoxic is proposed as a general expression to cover toxic, lethal and heritable effects to karyotic and extracaryotic genetic material in germinal and somatic cells’ (49). At this moment it is generally accepted that the carcinogenic capacity of neutrophils is at least partly attributable to the generation of DNA damaging and mutagenic ROS. However, it should be realized that neutrophilic ROS may be implicated in a wide variety of other processes that could all contribute to tumorigenesis (Figure 2).

In the past, a variety of genotoxic properties of phagocyte-derived ROS have been investigated, including their capacity to induce DNA damage, DNA mutations, and chromosomal aberrations. Before discussing the mechanisms by which neutrophils may cause oxidative damage to target cellular DNA,
some background information about the processes by which neutrophil-derived ROS per se may damage DNA is provided in the next paragraph.

**Modification of DNA by ROS**

A major development in carcinogenesis research in the past 20 years has been the discovery of DNA damage induced by ROS derived from both endogenous and exogenous sources (50). In the mid-1980s Ames and co-workers discovered that oxidized DNA bases were abundantly present in tissues of both humans and rodents (51). At the same time, it became evident that ROS-induced DNA damage was likely to be involved in carcinogenesis. Many of the ROS indeed have characteristics, which indicate that they are possible carcinogens (52) (also see Figure 2). Generally, the reactions contributing to ROS-induced DNA damage are oxidation, nitration, depurination, methylation and deamination. The reactivity of the various ROS towards the DNA is extremely variable. For instance, superoxide and hydrogen peroxide are thought not to react with DNA at all (52). The hydroxyl radical on the other hand, is the most DNA-reactive ROS, which generates a multiplicity of products from all four bases (53). The best-studied DNA lesion caused by hydroxyl radical attack is 8-hydroxydeoxyguanosine (8-OHdG), which is produced by a hydroxylation of the C-8 position of the guanine derivative of the DNA (54). Currently, there is a consensus that the presence of 8-OHdG, which is in fact a pre-mutagenic lesion, is associated with the carcinogenic process (55). In addition to 8-OHdG, numerous other kinds of OH-modified DNA bases have been described, including 5-hydroxymethyluracil, 5-hydroxyuracil, 5-hydroxyadenine, 8-hydroxyadenine and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (52). However, in contrast to 8-OHdG, the biological implications and significance of these lesions for carcinogenesis are often less well understood.

**Fig. 1.** Schematic overview of the generation and interaction of ROS produced by neutrophils. Three different pathways for the generation of *OH are indicated (A, B and C).

**Fig. 2.** Overview of a variety of processes by which neutrophil-derived ROS may promote tumour development. The present review specifically focuses on the processes involved in neutrophil-induced DNA damage and mutagenesis.
Although OH causes the most significant and abundant DNA base lesions in mammalians, other neutrophil-derived ROS may also interact with DNA. HOCl for instance, is shown to react with DNA, thereby inducing DNA–protein cross-links, chlorination of DNA bases, as well as pyrimidine oxidation products (56–58). One specific nucleobase damage product that is formed by treatment of naked DNA as well as cellular DNA with HOCl is 5-chlorouracil, which is also suggested to act as a fingerprint of DNA damage by HOCl (56,59,60). Owing to its extreme cytotoxic activity, HOCl-induced DNA damage in target cells is often associated with cell death. Therefore, the consequence of such DNA damage with regard to mutagenicity in competent proliferating cells is still controversial. Also peroxynitrite is suggested to damage DNA bases, probably by deamination or nitration (61,62). Neutrophil-derived ROS may also indirectly damage DNA through production of lipid hydroperoxides. In this process, a variety of very reactive side products can be generated, such as epoxides or aldehydes. Among these, malondialdehyde and 4-hydroxynonenal are extensively studied and are shown to be highly DNA reactive (63,64) and mutagenic (65). In addition to oxidizing DNA bases, ROS may cause DNA strand breakage owing to free radical attack of the DNA sugar-phosphate backbone (66).

Neutrophil-induced oxidative genotoxicity: evidence from co-cultures with target cells

In Table II, a historical overview of studies reporting oxidant-induced genotoxic effects of phagocytes, mostly of PMN, is listed. The general concept of these studies is that they all use co-cultures of phagocytes and specific target cells. In these experiments the phagocytic oxidative burst is generally initiated by addition of phorbol esters like phorbol-12-myristate-13-acetate (PMA) to trigger protein kinase C-mediated activation of NADPH-oxidase. A variety of genotoxic endpoints is then assessed in co-cultured bacteria or specific mammalian target cells. The first observation of phagocyte-induced genotoxicity, and the crucial role of ROS herein, was reported by Weitzman and Stossel in 1981 (67). They found that the ability of peripheral blood leukocytes to induce mutations in bacteria was markedly attenuated when cells were used from a patient with chronic granulomatous disease (CGD). In CGD, neutrophils have a defect in the NADPH-oxidase-mediated generation of O$_2^-$, and are, thus, unable to generate ROS. The first study on leukocyte-mediated genotoxic (clastogenic) effects in mammalian cells was published by Weitberg et al. in 1983 (69), describing leukocyte-induced sister chromatide exchanges (SCE’s) in hamster ovary cells. Further studies from Weitzman and Stossel (71) describing the induction of mutations in mammalian cells by phagocytes, finally, led to a publication from the same group showing the development of tumours in athymic mice injected with fibroblasts, which were previously exposed to activated human neutrophils (73). This was rather an important finding, since it provided a causal link between neutrophil activity, ROS release and carcinogenesis. Mechanisms were then further elucidated by the discovery of oxidative DNA base damage induced by neutrophils, first in naked DNA (87,88) and later also in target cellular DNA (79,83). Such oxidized DNA bases, including 8-OHdG are premutagic and could possibly explain the reported mutagenic effects of neutrophils. In other studies, neutrophils were also found to induce target cellular DNA strand breakage (see refs 75–77 and 86). Further crucial observations with regard to effects in the lung were published by Driscoll et al. (82,89), who demonstrated that neutrophils, obtained by broncho-alveolar lavage (BAL) from particle-exposed rats, were mutagenic to alveolar epithelial cells in vitro.

The specific role of ROS in phagocyte-induced genotoxicity was not only confirmed by using PMN from CGD patients but also by the use of a variety of specific antioxidants (72,90). This approach and, more specifically, the use of catalase, has led to a consensus that hydrogen peroxide is the major ROS that is mediating genotoxic processes induced by activated phagocytes (75,76,82,91). Indeed, by using various target cells in vitro, it was shown that reagent hydrogen peroxide was able to induce base damage and strand breakage in nuclear DNA (75,83,86,92,93). Furthermore, it has been established that the type of DNA base modifications, as detected in target cells exposed to reagent H$_2$O$_2$, is highly comparable with the damage induced by activated neutrophils. In general, these reflect hydroxyl radical-specific DNA modifications, indicating

<table>
<thead>
<tr>
<th>Target</th>
<th>Genotoxic endpoint</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Salmonella</td>
<td>Mutations</td>
<td>Weitzman and Stossel (67)</td>
</tr>
<tr>
<td>Photobact. fischeri</td>
<td>Mutations</td>
<td>Barak et al. (68)</td>
</tr>
<tr>
<td>Chinese hamster ovary cells</td>
<td>Sister-chromatid exchanges</td>
<td>Weitberg et al. (69)</td>
</tr>
<tr>
<td>Salmonella</td>
<td>Mutations</td>
<td>Fulton et al. (70)</td>
</tr>
<tr>
<td>Chinese hamster ovary cells</td>
<td>Mutations (HPRT)</td>
<td>Weitzman and Stossel (71)</td>
</tr>
<tr>
<td>Chinese hamster ovary cells</td>
<td>Sister-chromatid exchanges</td>
<td>Weitberg et al. (72)</td>
</tr>
<tr>
<td>Mouse fibroblasts</td>
<td>Malignant transformation</td>
<td>Weitzman et al. (73)</td>
</tr>
<tr>
<td>Human lymphocytes</td>
<td>Strand breaks and PARP activity</td>
<td>De Togni et al. (74)</td>
</tr>
<tr>
<td>Plasmodacytoma cell line</td>
<td>DNA strand breaks</td>
<td>Shacter et al. (76)</td>
</tr>
<tr>
<td>Tumour cells</td>
<td>DNA strand breaks</td>
<td>Chong et al. (77)</td>
</tr>
<tr>
<td>Human Ad293 cells</td>
<td>DNA strand breaks</td>
<td>Shacter et al. (78)</td>
</tr>
<tr>
<td>Mononuclear leukocytes</td>
<td>DNA base modifications</td>
<td>Dizdaroglu et al. (79)</td>
</tr>
<tr>
<td>Human Ad293 cells</td>
<td>DNA strand breaks</td>
<td>Van Staden et al. (80)</td>
</tr>
<tr>
<td>Alveolar epithelial cells</td>
<td>Mutations (frequency and spectrum)</td>
<td>Akman et al. (81)</td>
</tr>
<tr>
<td>Alveolar epithelial cells</td>
<td>Mutations (HPRT gene)</td>
<td>Driscoll et al. (82)</td>
</tr>
<tr>
<td>Chinese hamster ovary cell line</td>
<td>8-OHdG</td>
<td>Knaapen et al. (83)</td>
</tr>
<tr>
<td>Alveolar epithelial cells</td>
<td>Mutations (GPT gene)</td>
<td>Kim et al. (84)</td>
</tr>
<tr>
<td>Alveolar epithelial cells</td>
<td>DNA strand breaks</td>
<td>Knaapen et al. (85,86)</td>
</tr>
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</table>
that neutrophil-derived H₂O₂ acts as a progenitor of the hydroxyl radical, which then ultimately attacks cellular DNA (76,79,93,94). This is further illustrated by our own observations showing that activated neutrophils cause induction of hydroxyl radical-mediated 8-OHdG in pulmonary epithelial cells (83). In addition, other studies revealed that hydroxyl radical scavengers were effective in reducing neutrophil-induced sister chromatide exchanges (72), mutations (90) and DNA strand breakage (76) in target cells. Now, based on these collaborated findings it is hypothesized that neutrophil-derived H₂O₂, which is relatively stable, diffuses through the plasma membrane towards the nucleus where it reacts with DNA-bound transition metals to form hydroxyl radicals via the Fenton reaction. We observed that close contact between target cells and activated neutrophils is a prerequisite for this process to occur, as supernatants from activated neutrophils were unable to induce cellular DNA damage, probably since H₂O₂ is consumed by neutrophilic MPO or catalase (85). But even when close contact between neutrophils and target cells is allowed (e.g. in co-culture systems), there will be a constant balance between the consumption of H₂O₂ by MPO versus the capturing of diffusible H₂O₂ by the neighbouring target cells (95). This is possibly illustrated by recent experiments in which we observed that neutrophil-derived DNA strand breakage in co-cultured pulmonary target cells was increased upon inhibition of MPO activity, suggesting an increased availability of diffusible and DNA breaking H₂O₂ (86).

Although the studies as described above contribute to a concept of H₂O₂-induced genotoxicity by neutrophils, H₂O₂-independent mechanisms of neutrophil-induced genotoxicity should also be considered. For instance, Gera and Lichtenstein (96) demonstrated that neutrophil-derived defensins were able to induce DNA strand breaks in target cells. The possible role of neutrophil-derived ROS beside H₂O₂, is further illustrated by the observation that the mutational spectrum induced by activated neutrophils was different from the one induced by reagent H₂O₂ alone (81). One likely alternative candidate ROS would be HOCl. As described above, HOCl may induce a variety of mutagenic DNA base lesions, and studies have revealed the presence of HOCl-related DNA modifications in human and experimental animal inflammatory tissues (59,60).

Apart from inducing oxidative DNA damage, neutrophil-derived ROS have also been implicated in modifying DNA repair capacity of target cells. In vitro studies have for instance shown that neutrophils are able to inhibit poly(ADP-ribose) polymerase and DNA strand-break repair in neighbouring cells by virtue of their HOCl-generating capacity (97,98). Others showed that nitric oxide is a potent inhibitor of human Ogg1, a glycosylase that is involved in the repair of 8-OHdG lesions (99). Although these observations need to be confirmed in vivo, they support the concept that an inhibitory effect on DNA repair is an additional contribution of neutrophil-derived ROS to the mutagenic environment of chronic inflammation.

Oxidant-induced genotoxicity by neutrophils: evidence from in vivo studies

A large body of evidence for a role of neutrophils in pulmonary ROS-mediated genotoxicity has been derived from rat studies investigating toxicity of inhaled particles (for review see refs 14, 15 and 18). By the use of these animals, it has been demonstrated that chemical and physical differences between various poorly soluble particles (e.g. coal dust, carbon black, titanium dioxide) may not be the only factors predicting the lung tumour response, since patterns of tumour formation were not associated with any inherent genotoxic activity of such particles (100). This suggests that there must be a common mechanism by which high doses of both toxic (e.g. quartz, diesel soot) and non-toxic particles (e.g. titanium dioxide, carbon black) induce carcinogenesis in the rat. By considering such a mechanism, it is currently hypothesized that high particle doses induce a chronic inflammatory response, mainly caused by impairment of particle clearance by alveolar macrophages. Consequently, it is proposed that tumour formation is only found at particle doses eliciting a certain minimal level of phagocyte influx with associated ROS release, at levels that overwhelm pulmonary antioxidant and DNA repair protection systems. The constant release of ROS by activated inflammatory phagocytes is indeed assumed to be a crucial event, since ROS may cause genotoxic effects in the lung epithelium as described above. These genetic alterations, together with increased and chronic target cell proliferation and tissue remodelling, are now considered to be crucial components in the particle-induced carcinogenic response of the rat lung (9,18,100–102) (also see Figure 2).

The inflammatory response in a particle-exposed rat lung is characterized by an influx of neutrophils. For instance, in addition to an overall increase in inflammatory cells found in the BAL fluid, the percentage of neutrophils has been reported to increase from 3 to 4% in healthy non-exposed rats, up to levels >50% after acute or subchronic exposure to particles such as quartz, particulate matter or titanium dioxide. (82,89,103–106). Regarding a role of neutrophils in pulmonary genotoxicity, it has been established that in vivo mutagenicity in rat alveolar type II cells, isolated after particle exposure, was paralleled by a neutrophilic inflammation (82,89). Specifically, we and others demonstrated that the association between neutrophil numbers in BAL and HPRT mutation or DNA strand breakage in pulmonary epithelial cells shows a threshold, suggesting that endogenous protection mechanisms (e.g. DNA repair, antioxidant capacity) first need to be overwhelmed by neutrophil-derived ROS in order to cause significant genotoxicity (102,107). A further confirmation of the neutrophils’ role in particle-induced tumour formation in the rat was obtained by the observation that BAL-derived neutrophils from quartz-exposed rats were mutagenic (HPRT) to alveolar type II cells in vitro, whereas no direct effect of quartz was found in the same system. Notably, these in vitro effects of neutrophils were attenuated by the use of catalase, indicating that the crucial role of H₂O₂ in neutrophil-induced genotoxicity, as described above for in vitro co-cultures is also valid for the in vivo situation (82). Importantly, in the studies from Driscoll and co-workers, the capacity of alveolar macrophages to induce ex vivo mutagenesis was found to be significantly lower than the capacity of neutrophils, which is in line with observations describing an increased ROS-generating capacity of neutrophils compared with macrophages or monocytes (108,109). Further indications that neutrophils are indeed involved in the early stages of particle-induced carcinogenicity are found in studies showing that levels of DNA strand breakage, as well as 8-OHdG in lungs of acute and chronic quartz-exposed rats, were associated with the intensity of neutrophil influx (107,110–112).

Table III provides an overview of in vivo studies showing a relation between neutrophil influx and pulmonary genotoxicity in experimental animals. However, evidence obtained from
such particle-exposure studies is primarily associative, and no causal relationship between the presence of neutrophils and the induction of genotoxicity can be inferred from these studies. In fact, with respect to particle toxicology, there is still a lack of solid evidence that neutrophils are the true driving force behind particle-induced pulmonary genotoxicity in the rat. Indeed, in the last few years various contrasting results have been published. For instance, despite a massive neutrophil-influx, we did not observe increased 8-OHdG levels in rat lungs 7 days after exposure to silica. In parallel, we found a significant induction of APE/Ref-1, which led us to suggest that the absence of steady-state 8-OHdG increases under conditions of persistent neutrophil inflammation may result from a compensatory base excision repair (116). In another study, differential mutagenic effects were observed in rats exposed to crystalline or amorphous silica, both titrated at exposure levels to result in similar neutrophil influx. Despite these similar neutrophil ‘doses’, increased mutations in the HPRT gene were only found in rats exposed to crystalline silica (117). In addition, others observed that DNA strand breaks in BAL cells from carbon-black-exposed mice were independent of neutrophil infiltration (118).

Rather simple approaches to test the causality between neutrophil influx and pulmonary genotoxicity would be either to use animals that are systemically depleted from neutrophils, for instance by administration of anti-PMN antibodies, or to use animals in which pulmonary influx of neutrophils is prevented by depletion of alveolar macrophages or by treatment with anti-chemokine antibodies (113,119). At present, we are only aware of one single study that addressed pulmonary pathogenicity (unfortunately not genotoxicity) of particles in rats that were depleted from PMN. Interestingly, however, this study showed that the recruitment of PMN to the alveolar region is not necessarily linked with silica-induced pulmonary toxicity (120). In contrast, in a more recent and similar study, systemic depletion of neutrophils was shown to protect against airway pathogenicity induced after a 4 week inhalation challenge with LPS (121). Still, there is a lack of studies that have used such approaches to elucidate the causal role of neutrophils on pulmonary genotoxicity specifically. One crucial example is a study by Auten et al. (113), which showed that blocking of neutrophil influx by treatment with anti-ICAM antibodies caused a significant reduction of pulmonary oxidative DNA damage (8-OHdG and DNA nicking) in hyperoxia-exposed newborn rats. So far, this is the only study providing solid evidence for a causal role of neutrophils in eliciting pulmonary oxidative DNA damage in vivo. It is, however, questionable whether this approach, i.e. blocking neutrophil influx into the lung, can be applied in studies that focus on chronic effects of pulmonary neutrophilic inflammation. With regard to tumour formation for instance, it must be realized that neutrophils might play a dual role in later stages of the carcinogenic process. On the one hand PMN infiltration into tumours might stimulate tumour progression by facilitating tumour growth, metastasis and angiogenesis through the release of a variety of proteases and growth factors like vascular endothelial growth factor (1), as well as by contributing to genetic instability of the tumour cells through release of ROS (122). In line with this, it has been shown that tumour growth can be inhibited by the elimination of circulating PMN (123). On the other hand, PMN have also been identified as potent anti-tumour cells and studies suggest that neutrophil infiltration into some tumours is associated with a favourable prognosis (124). Paradoxically, this inhibitory role largely depends on the same weaponry of neutrophils, e.g. release of cell damaging ROS, which is assumed to mediate their tumour promoting activity.

Oxidative DNA damage by neutrophils: implications for the human situation

The major question to be answered is whether the concept of in vitro and in vivo neutrophil-induced pulmonary oxidative DNA damage and mutagenicity, as described above, can be extrapolated to humans. Current knowledge concerning the relation between neutrophils and pulmonary genotoxicity or carcinogenicity is largely obtained from high particle dose exposures using rats, which is recognized as a highly sensitive species with regard to particle-induced tumour formation. In humans, however, neutrophil influx is generally much less compared with (particle-exposed) rats: e.g. total cell numbers in the rat lung lavage can be increased >10-fold, with neutrophils exceeding 50%. In contrast, heavy smokers for instance show a total cell recovery in BAL of ~4–5× greater than from non-smoking healthy individuals, with increases of neutrophils up to 10–30% (125,126). In addition, in humans who are chronically exposed to crystalline silica, the inflammatory response in the lung is mainly characterized by an influx of lymphocytes and macrophages rather than neutrophils (9), suggesting that neutrophils only play a minor part. Nevertheless, more recent data on effects of pulmonary instillation of particulate matter in human volunteers indicate that neutrophils might have a role in the more acute phase of particle exposure, with BAL-neutrophil percentages up to 40% (127). However, even if such a profound neutrophil influx occurs in humans, it remains unclear whether this could lead to oxidant-induced genotoxicity. For instance, up to now there is still a lack of studies that tried to assess pulmonary oxidative DNA damage in lung diseases that are specifically characterized by neutrophil influx, such as chronic obstructive pulmonary disease or acute respiratory distress syndrome. An indication of the possible role of neutrophils in human pulmonary genotoxicity could be provided by previous studies from our own laboratory where we focussed on the nose as a more easily accessible part of the respiratory tract to evaluate respiratory neutrophil-induced DNA damage. To our own surprise, however, we could not find a relation between neutrophil numbers present in nasal lavage and 8-OHdG formation in nasal epithelial cells, although the presence of neutrophils was closely associated with epithelial cell proliferation (85). Although one should be cautious in interpreting these data, they indicate that the presence of neutrophils is not necessarily linked to oxidative DNA damage in the human respiratory tract.

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**Table III. In vivo studies showing an association between neutrophil influx and oxidant-related genotoxicity in the lung**

<table>
<thead>
<tr>
<th>Species</th>
<th>Exposure</th>
<th>Genotoxic endpoint</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>Quartz/carbon black (i.t.)</td>
<td>Mutagenesis (HPRT)</td>
<td>(82,89)</td>
</tr>
<tr>
<td>Rat</td>
<td>Quartz (i.t.)</td>
<td>DNA strand breaks</td>
<td>(107)</td>
</tr>
<tr>
<td>Rat</td>
<td>Quartz (i.t.)</td>
<td>8-OHdG</td>
<td>(111)</td>
</tr>
<tr>
<td>Rat</td>
<td>Quartz (i.t.)</td>
<td>8-OHdG, p53 mutations</td>
<td>(112)</td>
</tr>
<tr>
<td>Rat</td>
<td>Hyperoxia</td>
<td>8-OHdG</td>
<td>(113)</td>
</tr>
<tr>
<td>Rat</td>
<td>Carbon black (inhalation)</td>
<td>8-oxodG</td>
<td>(114)</td>
</tr>
<tr>
<td>Mouse</td>
<td>Diesel (inhalation)</td>
<td>8-oxodG, DNA strand breakage, (no increased mutagenesis)</td>
<td>(115)</td>
</tr>
</tbody>
</table>
would probably point to highly efficient antioxidant or DNA repair responses in humans, as opposed to the rat.

In considering the validity of the rat-neutrophil model of pulmonary genotoxicity for the human situation, assessment of species-specific host factors may provide useful information. In contrast to the rat, exposure of mice and hamsters to high lung particle doses has been much less associated with development of lung tumours, which is directly related to a less pronounced inflammatory response in these animals (15). Regardless of the actual numbers of neutrophils that enter the airways, the inherent capacity of neutrophils to generate ROS, as well as the differential ability of species and/or target cells to counteract the adverse effects of ROS may be further crucial factors in defining the resulting neutrophil-induced genotoxic effect. For instance, studies showed that the ROS releasing capacity of pulmonary neutrophils obtained from particle-exposed hamsters was much less in comparison with cells obtained from the rat (128). In addition, the hamster appeared to be far more effective at sustaining its anti-oxidant defences, suggesting a further explanation of the increased resistance of this species towards inflammation-induced carcinogenicity (129).

**Metabolic activation of chemical carcinogens by neutrophils**

By inhalation of polluted air (e.g. diesel exhaust, cigarette smoke, etc.) the human respiratory tract is exposed to a variety of environmental chemical carcinogens. Currently, it is largely accepted that in addition to the direct genotoxic effects of ROS, neutrophil-derived oxidants are also implicated in pulmonary genotoxicity via the promotion of metabolic activation of such inhaled chemicals, thereby generating highly DNA reactive and mutagenic compounds. For instance, neutrophils are able to transform aromatic compounds such as benzene into more carcinogenic metabolites through oxidant-mediated hydroxylation and nitration reactions (130). In addition, neutrophil-derived nitrite was associated with increased formation of mutagenic N-nitrosamines in the presence of nitrosatable amine precursors (131), whereas others showed that neutrophil-derived oxidants are able to activate heterocyclic amines such as 2-amino-3,8-dimethylimidazo[4,5-f]-quinoxaline (MeIQx) into mutagenic electrophilic products (132).

However, probably the most relevant pathway by which neutrophils may enhance metabolic activation of chemical carcinogenesis is a MPO-mediated peroxidative metabolism. A selection of studies showing neutrophil-mediated metabolic activation of chemical carcinogens is listed in Table IV.

At present there is accumulating evidence that MPO gene polymorphisms are associated with lung cancer risk (see refs 147–152). One of the most frequently occurring polymorphisms is a −463 G→A transition in the promoter region of MPO. Early studies showed that the presence of the A allele is associated with decreased MPO gene (mRNA) and protein expression in primary acute myeloid leukaemia cells, indicating a possible functionality of this polymorphism (153). Consequently, a number of case-control studies showed that persons carrying the mutant AA genotype have an up to 40–70% reduced risk for lung cancer (147–152), although other studies could not confirm these results (154,155). Nonetheless, since pulmonary MPO is largely derived from recruited neutrophils (156), these studies further suggest an involvement of neutrophils in human pulmonary carcinogenesis.

Considering the role of MPO in the generation of HOCl, it would be expected that the possible protective effect of the −463 A allele is mediated through a reduced production of HOCl. Although HOCl formation has been linked to induction of DNA damage *in vitro* and *in vivo*, as we discussed above, there is currently no evidence in support of a causal role of HOCl in pulmonary carcinogenesis. On the contrary, there is growing evidence that the protective effect of the A allele is predominantly observed in smokers, mainly suggesting an interaction between neutrophils, MPO and cigarette smoke constituents (149,150). This is further supported by data showing that the protective effect of the MPO polymorphism was found to be strongest for small cell lung cancer specifically, which is a type of lung cancer strongly associated with cigarette smoking (151). Cigarette smoke is a potent source of chemical carcinogens and, at present, it is believed that the association between MPO and pulmonary carcinogenesis can be explained by the involvement of neutrophil-derived MPO in metabolic activation of inhaled chemical carcinogens, including aromatic amines (157–159) and heterocyclic amines (133).

However, MPO activity has most frequently been linked to the metabolism of carcinogenic polycyclic aromatic hydrocarbons (PAHs) (126,141–144).

PAHs account for a large group of structurally related, widespread environmental pollutants that are formed during incomplete combustion processes. In the Western world, PAHs are produced by, for example, power plants and combustion engines. The most potent source for human exposure, however, is smoking. Benzo[a]pyrene (B[a]P) is the best studied PAH and has been widely used as a prototype PAH to reveal the relationship between metabolic activation processes and chemical carcinogenesis (160–163). Briefly, the activation of B[a]P involves a specific sequence of enzymatic reactions resulting in the generation of bay region diol epoxide (BPDE), which can form a covalent adduct with the exocyclic aminogroup of guanine in DNA (160,161–164). These BPDE–DNA adducts are mutagenic to bacteria as well as mammalian cells (165), elicit the transformation of cells in culture and are carcinogenic in several tissues including the lung (166,167).

**Activation of PAHs by neutrophil-derived MPO**

The capacity of neutrophils to metabolise PAHs to their DNA-binding metabolites was already described in the 1980s by *in vitro* and *in vivo* studies from Trush *et al.* (146) and Kensler *et al.* (145). In these studies, they found that the interaction of the B[a]P metabolite B[a]P-7,8-dihydrodiol with phorbol ester-activated neutrophils, but not alveolar macrophages (which lack MPO), resulted in the generation of a metabolite, which covalently binds to added calf thymus DNA. This effect could be ameliorated by the addition of the heme enzyme inhibitor sodium azide, suggesting the involvement of MPO (146). In addition, activated PMN were shown to enhance the mutagenicity of B[a]P-7,8-dihydrodiol in the *S. typhimurium* strain TA100 (146). These initial *in vitro* observations were also confirmed *in vivo*, by showing that recruitment and activation of PMN caused a 50% increase in covalent binding of B[a]P-7,8-dihydrodiol metabolites to epidermal DNA in the skin of CD-1 mice (145). The specific role of MPO was further revealed by showing that the B[a]P metabolite (±)B[a]P-7,8-diol may serve as a substrate for MPO, thereby generating (+)-anti-B[a]P diol epoxide (BPDE), which is the highly DNA reactive and most mutagenic metabolite of B[a]P (142,143).

Specifically, by stereochemical analysis of tetrod products it was shown that PMA-activated PMN, as well as an *in vitro* MPO–H₂O₂ system, primarily generated *anti*-dиеlepoxides.
Situation in the lung, support is provided by our observation of PAH-containing coal tar ointments (168). With regard to the respiratory tract (Figure 3).

In vivo neutrophils to genotoxicity, with special reference to the respiratory tract. Systemic depletion of neutrophils, or blocking neutrophil-influx into the lung by treatment with specific antibodies, seems to be a promising approach to further reveal the effect of neutrophils in animals acutely exposed to inflammatory agents (inhaled particles, micro-organisms, hyperoxia) (113). Such studies should ideally be performed by evaluating genotoxicity in target cells relevant for neoplastic outcomes, instead of evaluating DNA damage in whole lung homogenates obtained from mice exposed to bacteria by inhalation, were able to transform B[a]P-7,8-diol to a DNA-binding metabolite (143). More recently, these observations were confirmed by studies in our laboratory, showing that activated neutrophils enhance BPDE–DNA adduct formation in co-cultured alveolar epithelial target cells that were exposed to B[a]P-7,8-diol (144).

A major link between these mechanistic studies describing the role of MPO in the activation of chemical carcinogens, and the epidemiological studies indicating an association between MPO and pulmonary carcinogenesis in smokers, is possibly provided by a study in which we found a strong relationship between the MPO polymorphism and PAH–DNA adduct levels in skin of atopic dermatitis patients treated with PAH-containing coal tar ointments (168). With regard to the situation in the lung, support is provided by our observation that the MPO polymorphism was indeed associated with a reduced MPO enzyme activity in BAL of smoking individuals. This was accompanied by a significant reduction in PAH–DNA adduct levels in pulmonary cells (126). Since the formation of PAH–DNA adducts is considered as a crucial process in smoking-related carcinogenesis (169), these observations provide a first indication of the possible causal relation between neutrophils, MPO and pulmonary carcinogenesis in humans. Overall, these related studies further define a role of neutrophils in human pulmonary carcinogenesis in addition to their capacity to release ROS: they can enhance metabolism of environmental mutagenic carcinogens through release of MPO.

Neutrophils and pulmonary genotoxicity: gaps and needs

The association between chronic inflammation and the carcinogenic process has been observed for considerable time. However, the causal mechanisms that can explain this association are still unclear. In the current review, we have focussed on one possible candidate mechanism, i.e. the contribution of neutrophils to genotoxicity, with special reference to the respiratory tract (Figure 3). In vivo studies with rats have revealed a concept indicating that chronic neutrophilic inflammation upon high particle dose exposures may initiate and promote tumour growth in the lung. This seems to be largely facilitated by genotoxic processes through the release of DNA-damaging ROS, as well as by inducing cell proliferation. It should be clear, however, that the causal role of neutrophils in ROS-mediated DNA damage and mutagenesis is still primarily described by in vitro experiments. In vivo experiments using experimental animals have mainly provided indirect evidence, and additional research is needed to further test the relationship between neutrophils and ROS-induced genotoxicity in the respiratory tract. Systemic depletion of neutrophils, or blocking neutrophil-influx into the lung by treatment with specific antibodies, seems to be a promising approach to further reveal the effect of neutrophils in animals acutely exposed to inflammatory agents (inhaled particles, micro-organisms, hyperoxia) (113). Such studies should ideally be performed by evaluating genotoxicity in target cells relevant for neoplastic outcomes, instead of evaluating DNA damage in whole lung homogenates or in inflammatory cells obtained by BAL. We showed that isolation of pulmonary epithelial cells provides an opportunity to tackle neutrophil-induced in vivo genotoxicity in a ‘multipletarget-marker’ approach, since the contribution of various factors, including inflammatory cell influx, antioxidant status and cytotoxicity can be evaluated within a single animal (107).

Table IV. Examples of neutrophil-mediated or MPO-mediated activation of environmental chemicals into genotoxic metabolites

<table>
<thead>
<tr>
<th>Parent compound</th>
<th>Mutagenic/carcinogenic product or effect</th>
<th>Proposed mechanism</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amines</td>
<td>N-nitrosoamines</td>
<td>Nitration mediated by neutrophil NO-release (131)</td>
<td></td>
</tr>
<tr>
<td>N-aryldihydroxamic acids</td>
<td>Formation of 2-nitrosofluorene (2-NOF)</td>
<td>MPO-mediated peroxidation (133)</td>
<td></td>
</tr>
<tr>
<td>Aromatic amine (ABZ)</td>
<td>Formation of ABZ–DNA adducts</td>
<td>MPO-catalysed peroxidation reaction (134)</td>
<td></td>
</tr>
<tr>
<td>Aromatic amine (ABZ)</td>
<td>Formation of 3-Nitro-ABZ</td>
<td>Nitration reaction, catalysed by MPO in presence of nitrite (135)</td>
<td></td>
</tr>
<tr>
<td>2-Aminofluorene (2-AF)</td>
<td>2-AF–DNA adducts</td>
<td>MPO-catalysed (136)</td>
<td></td>
</tr>
<tr>
<td>Benzenes</td>
<td>Formation of Phenol, and p-nitrophenol</td>
<td>Peroxy nitrite-mediated hydroxylation and nitration reactions (130)</td>
<td></td>
</tr>
<tr>
<td>Heterocyclic amines (MeIQx)</td>
<td>Formation of electrophilic mutagenic products ( Ames test)</td>
<td>One electron oxidation mediated by ROS (132)</td>
<td></td>
</tr>
<tr>
<td>Heterocyclic amines (MeIQx)</td>
<td>Formation of N-nitroso compounds</td>
<td>MPO-catalysed nitration reaction (138)</td>
<td></td>
</tr>
<tr>
<td>Heterocyclic amines (IQ)</td>
<td>Formation of IQ–DNA adducts</td>
<td>MPO-catalysed (139,140)</td>
<td></td>
</tr>
<tr>
<td>Aromatic amine (3-ABA)</td>
<td>Formation of 3-ABA–DNA adducts</td>
<td>MPO-catalysed (141)</td>
<td></td>
</tr>
<tr>
<td>PAHs (B[a]P-7,8-diol)</td>
<td>B[a]P–DNA adducts</td>
<td>Peroxy/peroxide-dependent epoxidation reaction (142–146)</td>
<td></td>
</tr>
</tbody>
</table>

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The major question that remains to be answered is whether in vitro and in vivo observations in experimental animals can be extrapolated to humans. So far, it is still unclear whether neutrophils really cause ROS-induced genotoxicity in the inflamed human respiratory tract. On the other hand, recent molecular epidemiological studies in combination with experimental in vitro and in vivo studies have provided consistent evidence that neutrophils and human pulmonary carcinogenesis might be causally related by the capacity of neutrophils, via the release of MPO, to promote bioactivation of environmental carcinogens like PAHs. Nonetheless, a lot of work needs to be done to provide a deeper insight into the role of PMN in pulmonary DNA damage and mutagenesis and whether this could trigger pulmonary carcinogenicity. Such knowledge may eventually lead to new approaches for inflammatory cancer prevention.

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Neutrophils and respiratory track DNA damage


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