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Review

Role of Oxygen Free Radicals in Cancer Development

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In aerobic life, oxidative stress arises from both endogenous and exogenous sources. Despite antioxidant defence mechanisms, cell damage from oxygen free radicals (OFR) is ubiquitous. OFR-related lesions that do not cause cell death can stimulate the development of cancer. This review discusses the effects of oxidative stress at the different stages of carcinogenesis. Mutagenesis through oxidative DNA damage is widely hypothesised to be a frequent event in the normal human cell. A large body of evidence suggests important roles of OFR in the expansion of tumour clones and the acquisition of malignant properties. In view of these facts, OFR may be considered as an important class of carcinogens. Therefore, the ineffectiveness of preventive antioxidant treatments, as documented in several recent clinical trials, is surprising. However, the difficulties of antioxidant intervention are explained by the complexity of both free radical chemistry and cancer development. Thus, reducing the avoidable endogenous and exogenous causes of oxidative stress is, for the present, the safest option. In the near future, new insights in the action of tumour suppressor genes and the DNA repair mechanisms may lead the way to additional tools against carcinogenesis from OFR.

Key words: oxidative stress, mutagenesis, DNA repair, antioxidants, tumour promotion, p53, apoptosis
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INTRODUCTION

OXYGEN FREE radicals (OFR) are continuously generated in cells exposed to an aerobic environment. Antioxidant defence systems have co-evolved with aerobic metabolism to counteract oxidative damage from OFR [1, 2]. Despite the antioxidant defence, OFR-related damage of proteins [3] and DNA [4] accumulates during life and have been postulated to lead to such age-dependent diseases as atherosclerosis, arthritis, neurodegenerative disorders and cancer [5, 6]. A number of endogenous and exogenous cancer risk factors generate OFR *in vivo* [7, 11]. Therefore, there was much hope that the increase of cancer incidence in an ageing population could be reversed by removing avoidable sources of OFR and/or by enhancing the antioxidant defence systems [12].

In the recent years, convincing evidence has accumulated that OFR are indeed a relevant class of carcinogens [13–15]. Cancer development is now commonly recognised as a microevolutionary process that requires the cumulative action of multiple events [16]. These events occur in one cell clone and include in a simplified three-stage model: (1) the induction of DNA mutation in a somatic cell (initiation), (2) the stimulation of tumorigenic expansion of the cell clone (promotion) and (3) the malignant conversion of the tumour into cancer (progression). OFR can stimulate cancer development at all three stages, initiation [17], promotion [18] and progression [19]. In view of the ubiquity of OFR in aerobic organisms, this carcinogenic potential of OFR gives rise to concern. Alternatively it may open new perspectives for the prevention of cancer.

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AETIOLOGY OF OXYGEN FREE RADICALS

Chemistry of oxygen free radicals

Free radicals are molecules with one or more unpaired electrons. The reactive radicals responsible for tissue damage are generally short-lived species that are generated *in situ* [20]. Free radicals are produced in normal or pathological cell metabolism, from xenobiotics or through ionising radiation. An important feature of free radical reactions with non-radicals is that they result in new radicals, which leads to chain reactions [21]. Electron acceptors such as molecular oxygen react easily with free radicals, to become radicals themselves, the OFR. This explains why, in aerobic life, where molecular oxygen is ubiquitous, OFR become the primary mediators of cellular free radical reactions.

The first one-electron reduction of molecular oxygen produces the superoxide radical $\cdot O_2^-$ (Figure 1a). In aerobic metabolism, 1–2% of total consumption results in the production of superoxide [20]. *In vivo*, $\cdot O_2^-$ can act both as a reducing agent, as with

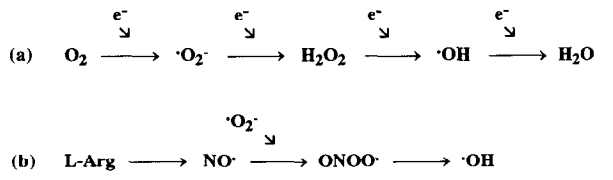

Figure 1. (a) Formation of superoxide radical ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$) and water by stepwise, univalent reductions of molecular oxygen. (b) Formation of nitric oxide (NO·) from L-arginine, and of peroxynitrite (ONOO·) and hydroxyl radical from nitric oxide and superoxide radical.

Fe^{3+} (see below), and as an oxidant, for example in the oxidation of thiol groups [20]. Although $\cdot O_2^-$ has relatively low reactivity and toxicity, it may function as an important second messenger in the cell [22]. However, at least a part of the biological effect of $\cdot O_2^-$ result from secondary products. The dismutation of $\cdot O_2^-$ yields hydrogen peroxide, H_2O_2. This reaction occurs spontaneously, or is catalysed by superoxide dismutases. The high reactivity of H_2O_2 *in vivo* is largely explained by the Fenton reaction, where H_2O_2 reacts with partially reduced metal ions such as Fe^{2+} or Cu^+, to form the hydroxyl radical ·OH. This reaction can be sustained *in vitro* by the presence of mild reducing agents such as $\cdot O_2^-$ or ascorbic acid, that recycle the oxidised metal ions. In contrast to H_2O_2, ·OH can directly inflict DNA damage [23]. ·OH is still seen as the most important radical in OFR-related cell damage, although it seems that other intermediates must be involved in the reactions that are commonly attributed to ·OH [24].

Besides the primary metabolites of molecular oxygen, $\cdot O_2^-$, H_2O_2 and ·OH, many other radical and non-radical molecules play an important role in mediating OFR-related effects *in vivo*. The reaction of OFR with biomolecules gives rise to organic radicals that can propagate the oxidative damage. Thus, the peroxidation of membrane lipids to organic peroxyl radicals initiates a chain reaction that may explain many membrane-mediated effects of OFR [20, 21]. The role of nitric oxide, NO·, in cellular redox reactions is increasingly acknowledged [25]. NO· may react with $\cdot O_2^-$ to form the reactive peroxynitrite anion, ONOO- and ·OH (Figure 1b) [26]. In contrast, it has also been shown that NO· can act as a chain-breaking antioxidant against lipid peroxidation [27]. Non-radical reactive oxygen metabolites include H_2O_2, HOCl, O_3 and the singlet oxygen $^1O_2$. The carcinogenic effects of these oxidants, when demonstrated, could in general be attributed to their high reactivity which produces free radicals *in vivo*. This is best illustrated by the example of H_2O_2, which gives rise to the mutagenic hydroxyl radical in the Fenton reaction [28].

Causes of oxidative stress involved in carcinogenesis

In aerobic life, OFR are formed in normal cell metabolism from molecular oxygen. Despite antioxidant defences, these OFR cause constant damage to oxidisable molecules, which are repaired or replaced in a dynamic equilibrium. Oxidative stress arises either from the overproduction of OFR or from the deficiency of antioxidant defence or repair mechanisms, and results in reversible or irreversible tissue injury. Examples of short-term oxidative stress are the ischaemia–reperfusion syndrome, acute inflammation and hyperoxia (e.g. hyperbaric oxygen). Given the long-term evolutionary development of cancer, these conditions, in contrast to chronic oxidative stress, are not expected to cause cancer, unless they are the source of primary mutagenic events.

An important endogenous cause of chronic oxidative stress is the inflammatory response [29]. Activated leucocytes generate $\cdot O_2^-$ and HOCl, that represent an important source of OFR *in situ* [30]. These OFR not only mediate the killing of the target cells but also induce oxidative stress in adjacent tissue cells. Activated neutrophils stimulate mutagenesis *in vitro* [31], and oxidative stress from chronic inflammation favours cancer development in many organs. It has been speculated that chronic inflammation contributes to one third of the world's cancer [6]. Cancer induction by chronic inflammation is frequently observed in ulcerative colitis [32]. Other examples of inflammation-related carcinogenesis are the mesothelioma caused by asbestos deposits [33] and urinary bladder cancer induced by *Schistosoma haematobium* infections [34]. The induction of hepatocellular carcinoma by viral hepatitis [35] is often cited in this context. Indeed, increased formation of oxidative DNA damage has been found in chronic hepatitis [36]. However, in this pathology, oxidative stress should be seen as a cofactor of the carcinogenesis stimulated by oncogene activation through DNA integration from the hepatitis B virus and by virus-mediated tissue necrosis and regeneration.

Important examples of exogenous causes of oxidative stress in our society and their carcinogenic consequences are shown in Table 1. Smoking, the major cause of bronchogenic carcinoma [37], chronically exposes the bronchial epithelium to OFR [38]. Oxidative stress from tobacco smoke arises: (1) through a potent mixture of reactive oxidants, in particular nitrogen oxides and the hydroxyl radical [39, 40], (2) through the depletion of the intracellular antioxidant glutathione (GSH) by aldehydes [41] and (3) through the induction of chronic inflammation [29]. OFR-related carcinogenesis is potentiated by the presence of several cocarcinogens in the tobacco smoke, including nitrosamines and polycyclic aromatic hydrocarbons, such as benzo(a)pyrene. The role of OFR in carcinogenesis from benzo(a)pyrene is 2-fold. First, OFR stimulate the metabolism of benzo(a)pyrene to diolepoxides that initiate tumours through the formation of DNA adducts [42]. Second, the metabolism of benzo(a)pyrene itself can generate H_2O_2 [43].

Both ultraviolet light and ionising radiation of higher energy (X-rays, γ-radiation) stimulate mutagenesis. The radiation induces DNA damage via the generation of OFR *in situ*, such as the hydroxyl radical, as well as from direct induction of free radicals in the biomolecules [44]. The involvement of oxygen-independent free radical reactions may explain a pattern of DNA lesions that is quantitatively different from that observed in other forms of oxidative stress. There is much evidence suggesting that

Table 1. Major exogenous causes of oxidative stress involved in carcinogenesis

Cause of oxidative stress	Oxygen free radicals	Cancer associated with exposure
Tobacco smoke	$NO\cdot_x$, ·OH	Bronchogenic carcinoma
Ultraviolet light	·OH, organic radicals	Melanoma and other skin cancer
Fatty acids in food	Lipid peroxides	Colorectal cancer, breast cancer
Iron and copper ions	·OH	Colorectal cancer
Ethanol	Lipid peroxides	Hepatocellular carcinoma, breast cancer

OFR from lipid peroxidation reactions are responsible for the association between fat intake and colorectal cancer [19, 45]. For fatty acids ingested with meat, iron (Fe^{2+}) is an important cofactor that enhances the production of OFR in the colon [46, 47]. Copper (Cu^{+}), that is as effective as iron as a catalyst in the Fenton reaction, is a more potent mutagen than iron in *in vitro* systems [48]. This may be explained by direct interactions of copper with the bases of DNA [13]. The epidemiological correlation between alimentary fat and breast cancer [37] is thought to result from carcinogenic lipid oxidation products in the breast fluid [49]. Ethanol is another major cancer risk factor that may in part act through free radicals generated during its metabolism. Thus it was speculated that OFR are involved in ethanol-related carcinogenesis, because the tumour-stimulating effect of ethanol was dependent on lipid peroxidation [50].

MECHANISMS OF OFR-RELATED MUTAGENESIS

Role of oxidative stress in tumour initiation

Initiation, the first step of carcinogenesis, requires a permanent modification of the genetic material in one cell. It is estimated that the number of oxidative hits to DNA is about 10 000 per cell per day in the human [6]. The DNA damage from OFR is continuously removed by specific and non-specific repair mechanisms [51]. Nonetheless, a very small part of the oxidative DNA lesions escapes repair and represents an important mutagenic potential that accumulates with age [4]. Higher doses of OFR increase the chance that the DNA lesions may not be effectively countered by DNA repair. Thus, exposure of mammalian cells to oxidative stress increases mutagenesis [52, 53]. However, an oxidative stress that is intense enough to kill cells becomes less effective in introducing DNA modifications in a cell population. These effects would result from a particular dose–effect relationship between OFR and cancer initiation through mutagenesis, where intermediate levels of oxidative stress are most effective (Figure 2). However, two points are important to note: (1) the borders of the dose-dependent effects in Figure 2 are not clear-cut: in an extreme case, one single OFR may cause cell death if it inactivates the single copy of an essential gene through a point mutation and (2) the biological effects of a given dose of oxidative stress critically depend on other parameters, such as the composition of the OFR involved, the presence of cocarcinogens, and the cell cycle position at the moment of exposure.

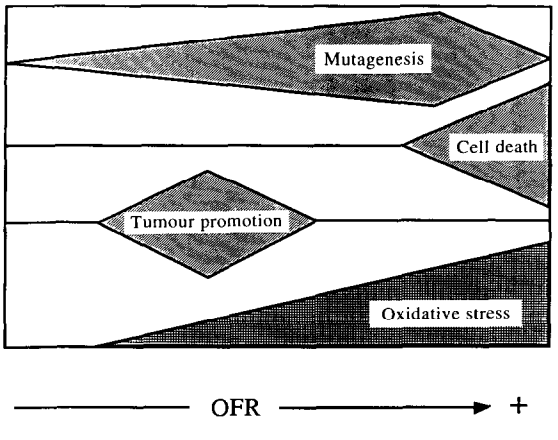

Figure 2. Hypothetical model of dose–response relationship between oxygen free radicals (OFR), oxidative stress and carcinogenic effects.

DNA base modifications

Oxidative DNA damage can be defined both chemically and structurally [54]. However, structural damage is always caused or accompanied by chemical DNA damage and, vice versa, every chemical modification entails some structural changes to the DNA double-helix. Nevertheless, DNA base modifications and DNA helix alterations will here be discussed separately. OFR-related base damage shows a characteristic pattern of modifications [23]. In various cancer tissues, an increase in OFR-specific DNA modifications was found [55, 56]. Most of these changes can be reproduced *in vitro* with systems that generate the hydroxyl radical, ·OH, for example the H_2O_2–Fe^{2+} ascorbic acid system [57] or hypoxanthine–xanthine oxidase (which generates the superoxide anion and H_2O_2) in the presence of iron ions [58]. This, however, does not imply that ·OH is the only free radical that mediates the mutagenic effect of oxidative stress *in vivo*.

The hydroxyl radical reacts with all components of the DNA molecule: the deoxyribose backbone, the purine bases and the pyrimidine bases [23]. The chemical alteration of the deoxyribose elements can cause the release of purine or pyrimidine bases, producing abasic sites which have been shown to be mutagenic *in vivo*. ·OH attack on the duplex DNA results in radical adducts with purine or pyrimidine bases that yield a variety of end products. A selection of characteristic products are shown in Figure 3. The ·OH adducts of adenine or guanine can result either in ring-fragmented bases such as the 5-formamido-4,6-diamino-pyrimidine (Fapy-Ade), or in hydroxypurines such as 8-hydroxy-guanine (8-OH-Gua). Examples of thymidine and cytosine products are thymine glycol (5,6-OH-Thy) and 5-hydroxy-cytosine (5-OH-Cyt), respectively.

While many of the OFR-related DNA base modifications result in a replicative block, some can induce point mutations by base misreading at replication. The most ubiquitous oxidative DNA base modification is 8-OH-Gua, occurring in approximately one in 100 000 guanidine residues in a normal human cell [59]. 8-OH-Gua can produce GC to TA transversions as a result of 8-OH-Gua–Ade mispairing. GC to TA transversions are frequently detected in the *RAS* oncogene [60] and represent a possible mechanism of tumour initiation by OFR. GC to TA transversions in the *TP53* tumour suppressor gene have been

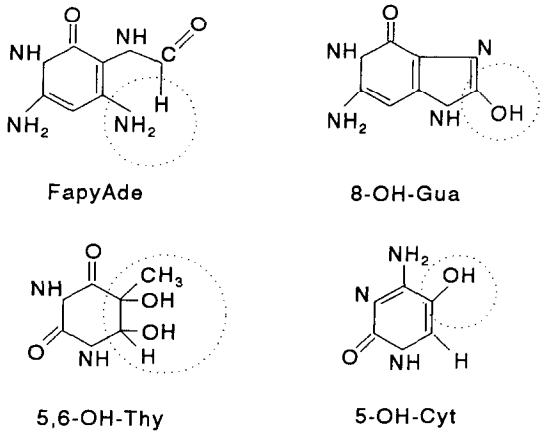

Figure 3. Characteristic DNA base modifications by OFR. 5-formamido-4,6-diamino-pyrimidine (FapyAde), 8-hydroxy-guanine (8-OH-Gua), thymine glycol (5,6-OH-Thy), and 5-hydroxy-cytosine (5-OH-Cyt) are derivates of adenosine, guanosine, thymidine and cytosine, respectively.

observed in lung and liver cancer [61, 62], and could be reproduced *in vitro* with an OFR-generating system [17]. Through the activation of oncogenes or the inactivation of tumour suppressor gene, these OFR-related point mutations may lead to initiation as a first step of carcinogenesis as well as participate in tumour progression at a later stage.

DNA helix alterations

Alterations of the DNA helix include helical distortion, single-strand breaks, double-strand breaks, interstrand crosslinks, and chromosomal aberrations [4, 23]. Major helix distortion can result from an additional bond between a base and the deoxyribose backbone, or from intrastrand purine dimerisation [4]. Intrastrand pyrimidine dimerisation is the major DNA alteration induced by ultraviolet light, while radiation of high energy results in higher proportions of DNA single- and double-strand breaks [44]. Single-strand [63] as well as double-strand breaks [64] may result from both direct free radical attack and from the OFR-related stimulation of enzymatic DNA cleavage. Modifications of the chromosomal structure can induce new, different structural DNA lesions in the replication process. The resulting genomic instability of the cell clone favours tumour progression and is a characteristic of cancer cells.

ANTIOXIDANT DEFENCE SYSTEMS

Antioxidant enzymes

The antioxidant enzymes superoxide dismutase (SD), catalase (CAT) and glutathione peroxidase (GPX) are the backbone of the cellular antioxidant defence system [2]. SD catalyses the dismutation of $\cdot O_2^-$ to H_2O_2. H_2O_2 is detoxified by CAT in a redox reaction that yields H_2O and O_2. GPX reduces both H_2O_2 and organic hydroperoxides using reduced glutathione (GSH) as the electron donor. In the absence of oxidative stress, cellular OFR are kept at a low level through the balanced, co-ordinated action of these three enzymes [65]. Both the decrease in antioxidant enzyme activity and the unbalanced overexpression of one of these enzymes, such as SD [29], can increase the vulnerability of mammalian cells to OFR.

Endogenous non-enzymatic antioxidants

Non-enzymatic antioxidants provide the primary defence against extracellular and intracellular OFR. They include a variety of lipophilic (bilirubin) and hydrophilic (GSH, uric acid) molecules or protein components (sulphydryl groups on cysteine) that act as free radical scavengers [2]. GSH, a tripeptide with a reactive sulphydryl group, acts on multiple levels of the antioxidant defence: (1) as a scavenger of free radicals such as $\cdot O_2^-$, $\cdot OH$ and lipid hydroperoxides [2], (2) as substrate for the antioxidant enzyme GPX and (3) in the direct repair of oxidative DNA lesions [66]. The action of uric acid, a purine base, is 2-fold: first, it is, like DNA itself, a substrate and scavenger of OFR; and second, by complexing iron ions, it prevents the generation of OFR in the Fenton reaction [67].

Exogenous antioxidant molecules

The role of small exogenous antioxidant molecules, such as α-tocopherol, β-carotene and ascorbic acid, has gained wide scientific and public interest. α-tocopherol, one of several isomers included in the term vitamin E, and β-carotene, a metabolic precursor of vitamin A, are lipophilic molecules. α-tocopherol effectively protects biological membranes from lipid peroxidation [2]. Thus, it may prevent membrane-mediated effects of OFR, that play a role in free radical chain reactions induced by lipid peroxidation, in Ca^{2+}-mediated apoptosis, and in Ca^{2+}-mediated tumour promotion. Although high α-tocopherol serum levels correlated with decreased cancer rates [68], the dietary supplementation of vitamin E plus β-carotene did not reduce the incidence of lung cancer in smokers over a 6 year period [69]. The cancer incidence in the β-carotene treatment arm of this trial was actually significantly increased by 18% [70]. In another trial, vitamin supplements that doubled β-carotene serum levels were without effects on the secondary incidence of non-melanoma skin cancer [71]. Negative results were also reported for β-carotene, vitamin C, and vitamin E, to prevent colorectal adenoma over a treatment period of 4 years [72].

Most of these studies, and a number of other antioxidant intervention trials that await completion, were based on successful animal models. However, major differences exist between human and animal antioxidant defence systems. Thus, ascorbic acid is an exogenous antioxidant for humans (vitamin C), but is produced endogenously in many mammalian species. Another important example is the high plasma levels of uric acid in humans as compared to most mammals, which may serve as a substitution for endogenous ascorbic acid [73]. Ascorbic acid is an effective hydrophil reductant and free radical scavenger *in vitro* and is thus supposed to afford antioxidant protection in both extra- and intracellular fluids. However, the effect of vitamin C on OFR levels *in vivo* is ambiguous. It has been shown that ascorbic acid, in concentrations equal or little above those found in human tissue, can replace the superoxide anion in the iron-catalysed Fenton reaction that produces the radical $\cdot OH$ through the reduction of H_2O_2. By this mechanism, it might actually increase OFR production from oxygen metabolites *in vivo* [74]. Vitamin C deficiency was associated with increased oxidative DNA damage in the human [75]. However, even in a vitamin-deficient population, the additional supply of vitamin C appears to have no effect on cancer incidence, either beneficial or harmful [76]. This finding is intriguing, given the high effectiveness of ascorbic acid in *in vitro* systems. One possible explanation is the auto-oxidative inactivation of 'excessive' concentrations of ascorbic acid in blood plasma [77]. Clinical trials that found an important prolongation of survival times in terminally ill cancer patients through intravenous treatment with very high doses of vitamin C [78] are today disputed by the majority of the scientific community. However, it must be pointed out that the reasoning behind that study was to boost the patient's immune system, rather than to inhibit OFR-related cancer progression.

DNA REPAIR MECHANISMS

The importance of the repair system for OFR-related DNA damage is dramatically illustrated in the human inherited xeroderma pigmentosum syndrome, where DNA repair defects impair the removal of pyrimidine dimers and other OFR-induced DNA lesions [79]. In these patients, the consequences of free radical attack on DNA are not limited to multiple skin cancers from ultraviolet radiation, but also entail a markedly increased susceptibility to several types of internal cancer [80].

The following summary of specific and general DNA repair mechanisms relevant to oxidative DNA damage is based on the thorough investigations that have been carried out in *E. coli*. In addition, some recent progress from the large research effort that focuses on the corresponding DNA repair systems in yeast and mammalian cells is mentioned. However, the mechanisms of the latter are in many cases only hypothesised from the

microbial models and much of the peculiarities of the eukaryote DNA repair systems remains to be explored [81, 82].

Repair of specific DNA lesions

OFR induce a variety of DNA base modifications, which, if not removed, can lead to mutagenesis. The roles of specific and general repair mechanisms in oxidative DNA damage were recently reviewed [51]. Inactivation of the specific DNA repair systems in *E. coli* substantially increases spontaneous and oxidative stress-related mutation rates. The particular importance of the OFR-related base modification 8-OH-Gua is underlined by a synergistic DNA repair system in *E. coli* that includes three gene products: (1) MutM, (2) MutY and (3) MutT. (1) The DNA glycosylase MutM initiates the repair of 8-OH-Gua sites and a variety of similar lesions in DNA by hydrolysing the corresponding base–deoxyribose bond. The detection of a corresponding repair enzyme in yeast suggests the existence of a eukaryote counterpart of MutM [83]. (2) 8-OH-Gua that escapes that repair mechanism has an important miscoding potential by pairing with adenosine, which leads to GC–TA transversions. MutY, a DNA mismatch glycosylase, recognises and removes the adenosine inserted opposite to the 8-OH-Gua. A mammalian homologue of MutY has recently been characterised [84]. (3) Finally, MutT hydrolyses 8-OH-GTP and thus removes it from the dNTP pool for DNA synthesis. This multilevel security against 8-OH-Gua-related DNA damage might explain how the intrinsic mutagenic potential of this frequent DNA lesion is kept at an acceptably low level [59]. Other *E. coli* endonucleases, with or without glycosylase activity, have been shown to been involved in the repair of oxidative DNA damage [51]. Thus, the DNA glycosylase endonuclease III recognises thymine glycol and a broad selection of other oxidative and non-oxidative base modifications. Several endonucleases without glycosylase activity recognise abasic sites resulting from direct free radical damage [85]. They were shown to be essential for the removal of the replication block induced by OFR-related single-strand breaks.

General repair mechanisms

The excision nuclease (exinuclease) system is the only repair system for removing bulky DNA adducts. Genes involved in the exinuclease system are uvrA, uvrB and uvrC in *E. coli* and the ERCC (excision repair cross complementing) group in humans [86]. Bulky DNA lesions include the pyrimidine dimers induced by ultraviolet light and other conditions that involve the generation of OFR. In addition to these DNA helix lesions, the *E. coli* exinuclease system also showed a weak repair activity on thymol glycol, 8-OH-Gua and abasic sites. The study of hereditary diseases associated with increased genetic instability has provided some exciting insights in the human excision repair system. Repair defects in the xeroderma pigmentosum syndrome affect the removal of pyrimidine dimers by the exinuclease system and were shown to include several genetic complementation groups [80]. The defective repair activity of OFR-induced DNA damage in extracts from xeroderma pigmentosa cells was found to be restored by *in vitro* complementation between the complementation groups A, B, C, D and G [79]. These data suggest that (1) OFR-induced DNA lesions are repaired by the same nucleotide excision-repair mechanism as the ultraviolet-induced pyrimidine dimers and (2) OFR-related DNA damage might contribute to the increased frequency of internal cancer in xeroderma pigmentosum.

A second major general DNA repair system is based on the recognition of base-pairing anomalies within the DNA helix [87]. The DNA mismatch repair system in *E. coli* genes includes the gene products MutH, MutL, MutS and MutU. For MutL and MutS, the human counterparts have been identified. Mutations in *HMSH2*, the human homologue of *MutS*, were shown to be responsible for the genetic instability observed in hereditary non-polyposis colon cancer (HNPCC) [88]. Interstrand crosslinks and double-strand breaks, that are not recognised by the exinucease or the mismatch repair systems, are among the major DNA helix alterations induced by oxidative stress. These, as well as any other OFR-related helix lesions, with the exception of chromosomal aberrations, are repaired by recombinational repair systems in *E. coli*. However, the details of the corresponding recombinational repair mechanisms in mammalian cells are not yet known [51].

While eukaryotic DNA repair mechanisms appear to include the elements that are found in bacteria, new mechanisms have evolved with the complex organisation of the chromosomes. Eukaryotic DNA is associated with histone and non-histone proteins that afford a relative protection against nuclease and free radical-mediated attack [51]. That protection is locally removed before DNA excision repair in mammalian cells takes place. Thus, DNA damage from OFR stimulates poly(ADP-ribose) polymerase to produce ADP-ribose polymers, that temporarily attract and detach histones from the DNA [89]. This mechanism is important for eukaryotic DNA repair, but may have additional implications. First, the resulting changes in DNA accessibility for transcription factors may represent a mechanism that regulates gene expression in response to oxidative stress. Second, in high levels of DNA damage, the NAD depletion by poly(ADP-ribose) polymerase becomes important enough to interfere with ATP synthesis. As ATP depletion can induce apoptosis, poly(ADP-ribose) polymerase activation may be an alternative pathway of OFR-related apoptosis [90]. Third, it has been speculated that the rapid depletion of NAD pools by poly(ADP-ribose) polymerase is a defensive mechanism to prevent an NADPH-driven Fenton reaction in oxidative stress [28].

ROLE OF OFR IN TUMOUR PROMOTION

In addition to the OFR-related mutagenesis involved in initiation and cancer progression, oxidative stress can stimulate the expansion of mutated cell clones by temporarily modulating genes related to proliferation or cell death. There is ample evidence that OFR can promote proliferation in mammalial cells after the initiation of cancer development by radiation or mutagenic chemicals [91]. While high levels of oxidative stress inhibit proliferation by cytotoxic effects, distinct low levels can stimulate cell division and promote tumour growth (Figure 2). The characteristic relationship between OFR levels and tumour promotion may explain why higher tolerance to oxidative stress (due to increased antioxidant enzyme activities) may lead to a promotable phenotype [92]. This example illustrates how OFR can selectively promote the growth of initiated cells, while having a toxic effect on the normal cell population. However, a more general role of OFR in tumour promotion was suggested with the hypothesis that chemical promotors, such as phorbol esters, act via the stimulation of intracellular OFR production [93].

Ca^{2+}-mediated tumour promotion

OFR can induce large increases in cytosolic Ca^{2+}, through the mobilisation of intracellular Ca^{2+} stores and through the influx

of extracellular Ca^{2+} [94]. The OFR-related changes in intracellular [Ca^{2+}] may regulate the transcription of genes involved in cell growth and proliferation through a direct effect of Ca^{2+} on the gene level, or through an indirect action. The induction of the proto-oncogenes *C-FOS* by low doses of OFR [95] was found to be directly mediated by cytosolic [Ca^{2+}] [96, 97]. An example of an indirect effect of OFR-related Ca^{2+} changes is the phosphorylation of transcription factors by Ca^{2+}-dependent protein kinases. The activation of PKC in oxidative stress was shown to be in part mediated by the OFR-related increase in intracellular [Ca^{2+}] [98]. The activation of PKC and other protein kinases leads to phosphorylation and activation of S6-kinase, which is involved in the acquisition of growth competence [99]. Both PKC and S6-kinase can regulate the activity of transcription factors via multiple phosphorylation cascades and may thus mediate many of the effects of OFR on cell proliferation.

Other mechanisms of tumour promotion by OFR

In addition to the Ca^{2+}-mediated regulation, OFR can directly modulate PKC activity through the oxidation of cysteine residues in the regulatory domain of the enzyme [100]. Poly ADP-ribosylation of chromosomal proteins in response to DNA damage [89] represents another mechanism that modulates gene expression in oxidative stress and is thought to be involved in tumour promotion. The expression of genes related to proliferation or cell death can also be directly modulated by the effects of intracellular redox levels on transcription factors. In bacteria, the direct oxidation of the oxy-R regulon is responsible for the transcriptional gene activation in response to oxidative stress [101]. In mammals, direct effects of OFR have been shown to regulate the activity of the transcription factor NF-kappaB, a member of the *rel* oncogene family [102]. A variety of genes, including growth and differentiation factors, are under the control of Rel proteins [103].

ROLE OF OFR IN CANCER PROGRESSION

The final stage in cancer development is the acquisition of malignant properties by the tumour. These properties include accelerated growth, escape from immune surveillance, tissue invasion and the formation of metastases. Most of these changes involve additional DNA lesions. It has been hypothesised that elevated OFR generation in tumour cells causes a persistent state of oxidative stress that increases genomic instability [104]. In addition, the sensitivity of tumour cells to OFR might be increased through lower activities of antioxidant enzymes [105]. However, as far as the mutagenic effects are concerned, the dose–response relationship between oxidative stress and tumour progression should be comparable to that described for tumour initiation (Figure 2). Nevertheless, these effects do not fully explain the role of OFR in cancer progression. First, tumour cells often display specific genetic changes that counteract OFR-mediated immune mechanisms by preventing apoptosis in response to oxidative stress. Second, cancer progression may be accelerated through increased genetic instability of tumour cells in oxidative stress.

Role of TP53 *mutations in OFR-related genomic instability*

Alterations in the *TP53* gene are among the most frequent mutations found in human cancer [106]. Experimental evidence suggests that the p53 protein is involved in cell cycle control. Normal cells respond to ionising radiation and other sources of OFR, which lead to the accumulation of DNA lesions, by a cell cycle arrest that allows DNA repair before replication. This effect correlates with an increase in p53 expression [107]. In contrast, cells lacking functional *TP53* proceed with cell divisions and thus permit DNA damage to be carried in the following generations. Thus, the unchecked cell division in the absence of *TP53* can cause continued chromosome rearrangement from the initial DNA damage. In view of these mechanisms, it is not surprising that *TP53*-deficient mice display greatly increased susceptibility to DNA damage from ionising radiation [108]. In the *TP53*-deficient animals, the frequency distributions of the different spontaneous and radiation-induced tumours are similar. As ionising radiation induces DNA damage via OFR, it has been hypothesised that a prime function of p53 is to protect against carcinogenesis from spontaneously generated OFR [108].

OFR and apoptosis

Most cancers stimulate an immune response of variable intensity in their host organisms. In terms of both the intensity of the response and the tumour cell susceptibility, OFR from activated leucocytes can cause: (i) chronic inflammation that does not eliminate the tumour cells, but enhances tumour progression; (2) apoptotic cell death through intracellular Ca^{2+} redistribution and other mechanisms [109] and (3) cell death from direct cytotoxicity. Only high, cytotoxic levels of OFR, that are of importance to tissue damage for the host organism, result in some elimination of tumour. In contrast, when OFR-production from the inflammatory cells is too low, and/or OFR-related cell death mechanisms are blocked in the tumour cell, the oxidative stress contributes to cancer progression by further DNA damage and growth stimulation.

The *BCL-2* proto-oncogene protects cancer cells from death through apoptosis [110]. *BCL-2* has been shown to inhibit apoptotic cell death induced by OFR [111]. Thus, it has been speculated that cancer cells which overexpress *BCL-2* may escape elimination by the immune system through their resistance against OFR-induced apoptosis. If the hypothesis is right, antioxidants might, similarly to *BCL-2*, interfere with the OFR-mediated elimination of tumour clones *in vivo* [112]. Thus antioxidant action could, instead of preventing carcinogenesis, stimulate tumour progression *in vivo*. This notion is important because it suggests that the *in vivo* situation might be fundamentally different from that in *in vitro* experiments, where antioxidants inhibited tumour progression [18].

CONCLUSIONS

OFR-related mutagenesis that can result in cancer initiation and progression is a frequent event in normal human cells. OFR-mediated tumour promotion has not been directly demonstrated in humans, but there is convincing experimental evidence that oxidative stress can differentially induce the proliferation of tumour cells. Thus, OFR should be recognized as an important class of carcinogens that stimulate cancer development at multiple stages. However, strategies to prevent the carcinogenic effects of OFR must take into account the complexity of both free radical chemistry *in vivo* and cancer development. Thus, the effect of oxidative stress at a given stage of carcinogenesis depends on the composition and intensity of the OFR involved. Therefore, the intervention with antioxidant enzymes that accelerate OFR conversion or with non-enzymatic antioxidants is a double-edged sword that might enhance the effect of the oxidative stress. However, even perfect protection against OFR by a well-balanced antioxidant action might actually stimulate cancer development through the improved survival of tumour cells.

In this light, the significant increase in lung cancer through the intervention with a free radical scavenger in a recent clinical trial [69] should be taken seriously. However, in most studies, supplementing antioxidants in a well-nourished population has not had a consistent effect on cancer incidence, either beneficial or harmful. One possible explanation for this apparent inefficacy may be that the follow-up was not sufficiently long to see the effects of antioxidants on tumour initiation by OFR-related mutagenesis. After cigarette smoking is begun, clinically demonstrable tumours occur with a mean lag time of more than 30 years [113]. This means that the follow-up of the current antioxidant intervention trials might simply not have been long enough to detect the effect on cancer incidence.

The great diversity of OFR-related DNA damage is reflected by the corresponding repair mechanisms that have been found in aerobic life forms. However, more knowledge on the biochemistry of mutagenesis and on the DNA repair mechanisms in eukaryotes is needed before interventions to support the repair system could be designed. In contrast, the involvement of oncogenes at all stages of OFR-induced carcinogenesis already evokes the possibility of gene therapy. Thus, reconstitution of the mechanisms that mediate cell cycle arrest in response to DNA damage and/or oxidative stress might be used to slow OFR-related tumour progression [114, 115]. Currently, the reduction of avoidable endogenous (i.e. chronic inflammation) and exogenous sources of oxidative stress and the elimination of environmental cocarcinogens represent potentially the most important means of preventing OFR-related cancer.

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