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Non-irradiation-derived reactive oxygen species (ROS) and cancer: therapeutic implications

Review Article

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Summary. Owing to their chemical reactivity, radicals have cytocidal properties. Destruction of cells by irradiation-induced radical formation is one of the most frequent interventions in cancer therapy. An alternative to irradiation-induced radical formation is in principle drug-induced formation of radicals, and the formation of toxic metabolites by enzyme catalysed reactions. Although these developments are currently still in their infancy, they nevertheless deserve consideration. There are now numerous examples known of conventional anti-cancer drugs that may at least in part exert cytotoxicity by induction of radical formation. Some drugs, such as arsenic trioxide and 2-methoxy-estradiol, were shown to induce programmed cell death due to radical formation. Enzyme-catalysed radical formation has the advantage that cytotoxic products are produced continuously over an extended period of time in the vicinity of tumour cells. Up to now the enzymatic formation of toxic metabolites has nearly exclusively been investigated using bovine serum amine oxidase (BSAO), and spermine as substrate. The metabolites of this reaction, hydrogen peroxide and aldehydes are cytotoxic. The combination of BSAO and spermine is not only able to prevent tumour cell growth, but prevents also tumour growth, particularly well if the enzyme has been conjugated with a biocompatible gel. Since the tumour cells release substrates of BSAO, the administration of spermine is not required. Combination with cytotoxic drugs, and elevation of temperature improves the cytocidal effect of spermine metabolites. The fact that multidrug resistant cells are more sensitive to spermine metabolites than their wild type counterparts makes this new approach especially attractive, since the development of multidrug resistance is one of the major problems of conventional cancer therapy.

Keywords: Radicals – Reactive oxygen species (ROS) – Polyamines – Bovine serum amine oxidase – Cancer

Abbreviations: BSAO, bovine serum amine oxidase; CuAO, copper-containing amine oxidase; DAO, diamine oxidase; GSH, glutathione; GSSG, oxidised glutathione; MAO, monoamine oxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; TPQ, 2,3,5-trihydroxyphenylalanine (cofactor of serum amine oxidases)

Introduction

The term reactive oxygen species (ROS) encompasses the non-radical species hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), the radicals superoxide anion ($O_2^{\bullet -}$) and the hydroxyl radical ($HO^{\bullet}$). Owing to their reactivity free radicals are cytotoxic. Their formation by irradiation is beside surgery the most frequent therapeutic intervention for the elimination of cancer cells. After the presence of ROS and related reactive species had been shown in biological material (Commoner et al., 1954), not surprisingly their cytotoxic potential was in the foreground of interest, and they were nearly exclusively considered to be the cause of undesired cellular damage and aging (Harman, 1956), against which the organism had to be defended. In agreement with this view, the focus of interest has been on the identification and advancement of compounds which are either radical scavengers or antioxidants. These were expected to prevent detrimental effects, e.g. in pathological situations, which are linked to excessive ROS formation, or an imbalance of redox regulation. Oxidative stress is indeed a frequent complication of various disease conditions: for example, ROS are potential carcinogens, because they facilitate mutagenesis and promote tumour progression. ROS have also been implicated among others in diabetes, atherosclerosis, rheumatoid arthritis, HIV and several neurodegenerative diseases.

Stimulated by the discovery of cell signalling and other functions by nitric oxide ($NO^{\bullet}$) (Lane and Gross, 1999; Wink et al., 1996), an intrinsic radical, the assessment of free radical functions has profoundly changed. It became obvious that ROS are involved in the physiological regulation of a wide range of signalling pathways, including apoptosis signalling (Dröge, 2002). Short-lived radicals are intracellular signals, owing to its relatively long half life and its ability to cross membranes. In contrast, H_2O_2 mediated signal transduction may function even between cells, and the membrane receptor may function at the same time as sensor of extracellular signals and as sensor of signals for the intracellular metabolic state.

Now, 50 years after the detection of free radicals in biological material, another development appears to gain shape: the rational use of ROS formation in therapy. This is the topic of the present review. Emphasis will be on the targeted formation of H_2O_2 by enzymatic oxidative deaminations as a promising approach in cancer therapy.

Sources and metabolism of reactive oxygen species

The mitochondria are the major, though not exclusive source of endogenous ROS. The electron transport chain leads to the formation of superoxide radical ($O_2^{\bullet -}$), H_2O_2, and the hydroxyl radical ($HO^{\bullet}$). The superoxide radical is formed from molecular oxygen by reduction. This reaction is catalysed by NAD(P)H oxidases and by xanthine oxidase. Non-enzymatically it is formed by reduction of O_2 with suitable redox-reactive compounds, such as the semi-ubiquinone/ubiquinone redox pair of the mitochondrial electron transport chain (Fig. 1).

In the mammalian organism H_2O_2 has a central position within the ROS family. Its formation by several reactions and its controlled inactivation is the basis of ''redox homeostasis'' (Chance et al., 1979). In Fig. 2 the metabolic interrelationships of H_2O_2 are shown. $O_2^{\bullet -}$ forms H_2O_2 by a reaction that is catalysed by the superoxide dismutase (SOD), a mitochondrial Mn^{2+}-containing enzyme. In addition a variety of oxidases, which use molecular oxygen as substrate form H_2O_2: enzymes with FAD

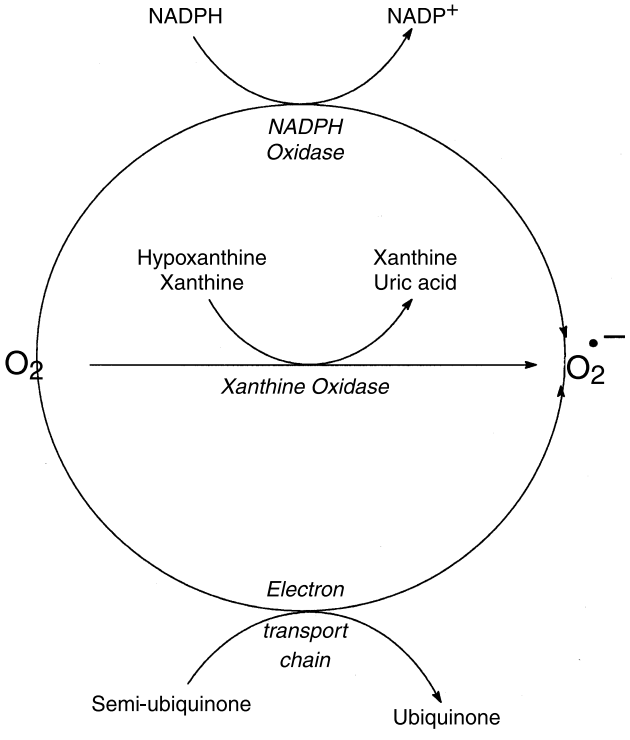

Fig. 1. Pathways of mitochondrial superoxide formation

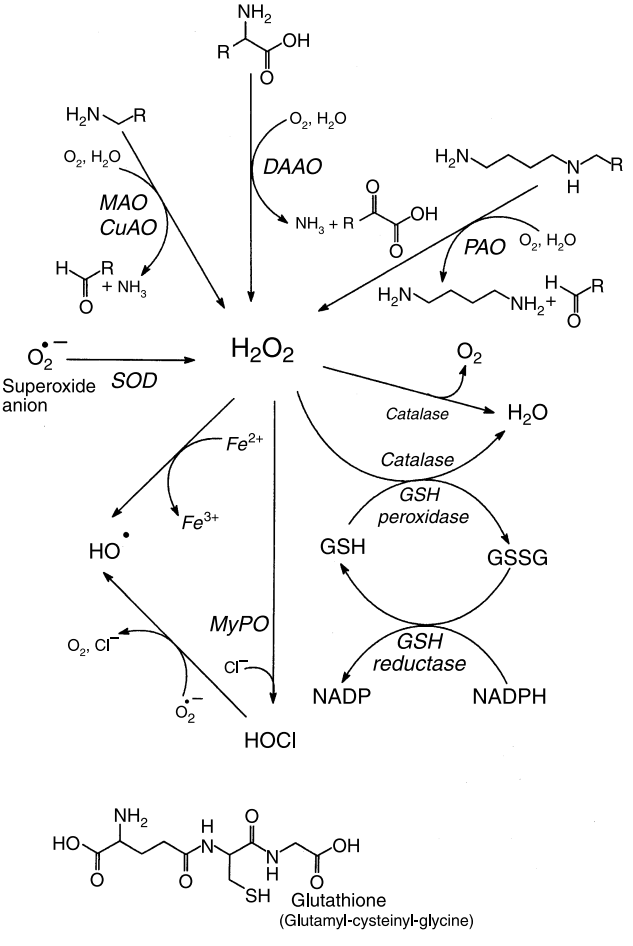

Fig. 2. Metabolic reactions of hydrogen peroxide. CuAO copper-containing amine oxidase, DAAO D-aminoacid oxidase, GSH glutathione, GSSG glutathione (oxidised form), MAO monoamine oxidase, MyPO myeloperoxidase, PAO polyamine oxidase, SOD superoxide dismutase

as cofactor (e.g. monoamine oxidase (MAO), polyamine oxidase (PAO), D-amino acid oxidase (DAAO)), copper-containing amine oxidases (CuAOs) (e.g: diamine oxidase (DAO), serum amine oxidases, lysyl oxidases, glucose oxidase, xanthine oxidase and peroxidases). Enzymes of the arachidonic acid pathway (cyclooxygenases, lipooxygenases) also contribute to ROS formation.

H_2O_2 is the source of the highly reactive hydroxyl radical and of hypochlorous acid, a product of the lysosomal enzyme myeloperoxidase (Fig. 2). From hypochlorous acid the superoxide anion and singlet oxygen may be formed (for a detailed description of the sources of ROS and the enzymes involved in their metabolism see Beaudeux and Vasson, 2005).

H_2O_2 is mainly decomposed by two enzymes of the cellular defence system: glutathione peroxidase and catalase. Both enzymes catalyse the oxidation of glutathione (GSH) to a dithiol (GSSG). In the absence of GSH or other substrates catalase releases molecular oxygen from H_2O_2 (Fig. 2). The GSH content constitutes the largest component of the endogenous redox buffer system. Hence GSH and some other thiols (cysteine, thioredoxine) are the major elements of the redox control system. They protect against excessive ROS and determine the sensitivity of cells to oxidative stress. In addition to enzymatic transformations of the thiols, antioxidants that are present at low concentration in cells, compete with other oxidisable substrates (proteins, lipids etc.) and prevent or delay their oxidative damage. To these belong α-tocopherol (vitamin E), β-carotene, and ascorbic acid (vitamin C). But amino acids, particularly cysteine and the aromatic amino acids may also contribute to the ROS scavenging activity of mammalian cells. The natural polyamines putrescine spermidine and spermine are also radical scavengers and metal chelators (Lovaas, 1997).

Reactive oxygen species and cancer

Several reviews were recently published with the aim to re-assess the role of free radicals in cancer, or to emphasise the function of free radicals in cellular signalling cascades (Turpaev, 2002; Das, 2002; Black, 2004a; Storz, 2005).

As long as ROS were exclusively considered as harmful products of cell metabolism, and not, as now compelling evidence suggests, being physiological components of numerous signalling cascades (Dröge, 2002), interrelationships between ROS and malignant growth appeared plausible, and it was not too surprising that several observations suggested mutagenic effects of ROS (Dreher and Junod, 1996; Hussain et al., 1994; Nakamura et al., 1988; Salim, 1993). However, a large body of evidence supported at the same time a pivotal role of excessive ROS production in senescence (Dröge, 2002). Therefore it is unlikely that the increase in cell proliferation rate of normal cells due to elevated ROS pools (Burden, 1995) is sufficient to induce malignant transformation. All ROS-related approaches to carcinogenesis suffer from this contradiction. It prevented the formulation of an unambiguous concept that explains both senescence and malignant transformation on the basis of enhanced ROS formation.

As is well established, carcinogenesis is a multistep process that requires the participation of several endogenous and exogenous factors. The chemical modification by radical-induced reactions of the pyrimidine and purine bases, and particularly the damage of the deoxyribosyl backbone of DNA by ROS is critical. Critical are also excessive transformations of other essential cell components (peroxidation of lipids, oxidative damage of proteins, including enzyme proteins). As pointed out by Valko et al. (2004), DNA lesions are genotoxic. The formation of 8-HO-G is known to be easily achieved and is mutagenic; it involves GC – TA transversions. In addition ROS production induces, mainly via MAP protein kinases and several transcription factors (AP-1, ATF, NF-κB etc.), the expression of a number of genes, such as H-Ras^{v12} and mox 1, which maintain the malignant phenotype (Irani et al., 1997; Su et al., 1994; Storz, 2005). In contrast, normal cells show an increased proliferation rate, and the expression of growth-related genes (c-fos, c-myc, etc.), if exposed to H_2O_2 or $O_2^{\bullet -}$ (Burden, 1995; Crawford et al., 1988). Furthermore the cellular defence systems against ROS damage are up-regulated, implying the up-regulation of gene products that can alter cell biology, and include anti-apoptotic factors, such as anti-apoptotic members of the Bcl-2 family, and inflammatory proteins (Greenberger et al., 2001).

A variety of tumour cells, among these multidrug resistant cells (P-glycoprotein expressing phenotype) produce very high amounts of ROS (see e.g. Szatrowsky and Nathan, 1991; Li et al., 1990; Soares et al., 1994; Arancia et al., 2004), and in vivo many tumours appear resistant to oxidative stress and apoptosis. Vaquero et al. (2004) suggested even a pro-survival, anti-apoptotic function of ROS in pancreatic cancer cells, which they relate to the therapy resistance of this cancer. According to Pelicano et al. (2004) the escalated ROS generation in cancer cells serves as an endogenous source of DNA-damaging agents, which promote genetic instability and development of drug resistance. The intrinsic oxidative stress of cancer cells appears

associated with the up-regulation of SOD and catalase expression (Hileman et al., 2004). On the basis of an increased ROS production in cancer cells therapeutic strategies have been suggested, which rely on the assumption that cancer cells, mainly multidrug resistant cells, are more sensitive to additional exposure to radicals than normal cells (Calcabrini et al., 2002; Pelicano et al., 2004; Hileman et al., 2004). In agreement with this suggestion is the fact that numerous established anticancer drugs generate ROS (or other radicals) as is briefly discussed in the following section. As suggested by Arancia et al. (2004), it is likely that the apoptotic effect of these drugs is due to the additional push of radical formation, which accompanies the apoptotic cascade.

Drugs inducing ROS formation

a) Conventional anticancer drugs trigger ROS formation

Apoptosis induction via death receptors (e.g. APO-1 Fas (CD95)) frequently triggers ROS formation (see e.g. Johnson et al., 1996; Um et al., 1996), although this seems not a general prerequisite of programmed cell death, since APO-1/Fas ligand activation is not always accompanied by ROS production (Hug et al., 1994). But in general high ROS concentrations induce apoptosis in a variety of cell types (Slater et al., 1995). Therefore, perhaps not surprisingly, more and more established anticancer drugs have been found to induce an enhanced formation of ROS. The therapeutic efficacy of these drugs (but also their side effects) may at least in part depend on ROS production.

The increase of ROS levels following polychemotherapy was demonstrated by the determination of thiobarbituric acid – reactive substances in the plasma of patients (Look, Musch, 1994). In Table 1 examples of ROS formation-triggering drugs are compiled. This list is certainly incomplete. The recent systematic exploration of the apoptotic signalling pathways for targets suitable for anti-cancer therapy (Fulda and Debatin, 2004; Cummings et al., 2004) will reveal many more examples. A detailed discussion of these drugs is beyond the frame of this review; however, two examples should be briefly introduced, because in these cases ROS formation is intentionally employed as a therapeutic principle.

Table 1. ROS formation triggering anti-cancer drugs

Drug	Reference
Actinomycin D	Ikeda et al. (1999)
Anthracyclines	Efferth and Oesch (2004)
Adriamycin	Bounias et al. (1997)
Doxorubicin	Benchekroun et al. (1993)
Farmorubicin	Bounias et al. (1997)
4-Epiadriblastin	Look and Musch (1994)
Arsenic trioxide	Gupta et al. (2003)
Artemisinins	Efferth and Oesch (2004)
Bleomycin	Dorr (1992); Guedez and Zucali (1996); Khadir et al. (1999); Seidel et al. (2003); Hong et al. (2003); Lee et al. (2004a)
Capsaicin	Ito et al. (2004)
Cisplatin	Olas and Wachowicz (1998)
Cyclophosphamide	Kanekal and Kehrer (1994)
Hydroperoxide derivative of cyclophosphamide	Murata et al. (2004)
Cytokines	
Tumour necrosis factor-α (TNF-α)	Thomas et al. (2002)
Cytosine arabinoside	Kanno et al. (2004); Iacobini et al. (2001)
Endotoxins	
E. coli Lipopolysaccharide	Victor and De la Fuente (2003)
Etoposide	Gantchev and Hunting (1997)
Fatty acids (polyunsaturated)	
γ-Linoleic acid (Li salt)	Ilc et al. (1999)
Docosahexaenoic acid	Sturlan et al. (2003)
Hydrazine type anticancer drugs	
Procarbazine*	Goria-Gatti et al. (1992); Ogawa et al. (2003)
2-Methoxyestradiol	Huang (2000)
*Quinone type anticancer drugs***	Handa and Sato (1975); Halinska et al. (1998)
Mitomycin C	Bounias et al. (1997); Lee et al. (2004b)
Sodium selenite	Li et al. (2003)
Vincristin	Look and Musch (1994)

* Forms methyl radicals
** Form semiquinone radicals

b) Estrogens

Mitochondrial SOD is considered to have a key role in the defence of aerobic cells against oxidative stress. Its inhibition causes the accumulation of $O_2^{\bullet -}$, but prevents H_2O_2 formation as is obvious from Fig. 2. From the accumulation of $O_2^{\bullet -}$ an increase of the oxidative damage and a new strategy of anticancer treatment was expected (see e.g. Hileman et al., 2001; Pani et al., 2004). The issue is, however, more complicated than was presumed: overexpression of SOD paradoxically inhibited growth of a wide variety of tumour cells (Oberley, 2005). Furthermore, mimics of SOD, such as tetrakis(4-benzoic acid) porphyrin and related metalloporphyrins increased H_2O_2 levels, inhibited the proliferation of normal cells, and killed tumour cells (Ohse et al., 2001), particularly well in com-

bination with cytotoxic compounds (Laurent et al., 2005). The conclusion from these and related observations was that H_2O_2, a product of SOD, is presumably the major effector molecule, from which more reactive radicals are formed than from $O_2^{\bullet -}$. This idea is supported by the fact that catalase and glutathione peroxidase modulate the effect of SOD over-expression. Nevertheless, 2-methoxyestradiol entered clinical testing as a presumptive inhibitor of SOD, even though it turned out that it does not inhibit SOD (Kachadourian et al., 2001). 2-Methoxyestradiol enhances the formation of ROS and induces apoptosis in leukaemia cells by a free radical-mediated mechanism (Huang et al., 2000). Amplification of $O_2^{\bullet -}$ formation by the xanthine oxidase reaction, or by other radical forming interventions increases cell death, and anti-tumour efficacy of 2-methoxyestradiol (Lambert et al., 2002). Leukaemia cells with a high basal $O_2^{\bullet -}$ content are more sensitive to 2-methoxyestradiol than cells with a low basal $O_2^{\bullet -}$ content. Arsenic trioxide, an inducer of ROS formation (see below) significantly improves the cytotoxicity of 2-methoxyestradiol (Zhou et al., 2003).

Indirect evidence for a receptor-mediated formation of ROS by estrogens is available for estrogen receptor-α positive (MCF-7) breast cancer cells. Upon exposure to 17-β-estradiol these cells showed a marked sensitivity to oxidative damage of DNA, and the decrease of their ability to detoxify ROS was found to be due to a loss of catalase activity and GSH. These changes were paralleled by the increase of the antioxidant activity-related enzymes SOD and glutathione peroxidase. The estrogen effects were antagonised by tamoxifen, the well known estradiol receptor-antagonist. In contrast, MDA-MB-231 cells, which lack the estradiol receptor, did not show any of these effects, suggesting that the sensitivity of breast cancer cells to oxidative damage is receptor mediated (Mobley and Brueggemeier, 2004).

c) Arsenic trioxide

Arsenic trioxide (As_2O_3) the notorious poison of classic crimes found in recent years a revival as a safe and efficacious treatment of promyelocytic leukaemia. It reacts with water reversibly to form $HAsO_2$ and H_3AsO_3. ROS formation is an essential mechanism of As_2O_3-induced apoptosis (Gupta et al., 2003). Exposure to As_2O_3 is accompanied by the characteristic biochemical and morphological changes of programmed cell death (activation of caspase-3, release of cytochrome c, down-regulation of Bcl-2 and Bcl-X_L, cell shrinkage, oligonucleosomal DNA fragmentation etc.) (Akao et al., 2000; Ora et al., 2000; Gupta et al., 2003). In agreement with ROS-induced cell death is the resistance to As_2O_3 of cells with a high GSH content. Resistance develops at least in part due to the up-regulation of the GSH pool (Davison et al., 2004). GSH depletion by inhibition of its synthesis (e.g. by buthionine sulfoximine) overcomes resistance (Davison et al., 2003). The activation of c-jun terminal kinase (JNK) appears essential for signalling of As_2O_3-induced apoptosis, since the sensitivity of cells to As_2O_3 decreased by exposure of acute promyelocytic leukaemia cells to dicumarol, an inhibitor of JNK (Davison et al., 2004).

Methyl-derivatives of As_2O_3 are even more active apoptotic inducers than As_2O_3. Hepatic (HepG2) cells are able to methylate As_2O_3, and release the methyl-derivatives, which are preferentially taken up by NB4 cells, a promyelocytic leukaemia cell line (Chen et al., 2003). It is not excluded that in vivo the hepatic methylation contributes to the anti-leukaemia effect of As_2O_3.

In view of the success of As_2O_3 in the treatment of promyelocytic leukaemia, several attempts were made to extend its application to a wider range of malignant cells with a higher resistance to this drug, by combining it with compounds that are capable of inducing ROS formation. Examples are docosahexaenoic acid (Sturlan et al., 2003), emodin (a natural anthraquinone derivative) (Yi et al., 2004), and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a widely used antioxidant (Diaz et al., 2004). These compounds appear to synergise the anti-tumour effect of As_2O_3 by generating ROS, but protecting non-malignant cells from As_2O_3-mediated oxidative damage.

d) The two faces of antioxidants

Antioxidants have been proven to antagonise oxidative stress by competing with other substrates of ROS. An instructive example for the functioning of antioxidants is the following: β-Lapachone, a topoisomerase I inhibitor and potent anticancer drug induces apoptosis in cell lines with low GSH content (e.g. HL-60 human promyelocytic leukaemia cells), but much less effectively in cells with a high GSH content. Induction of apoptosis is accompanied by an elevation of H_2O_2 formation. The antioxidants N-acetylcysteine, ascorbic acid, α-tocopherol, but not 2-mercaptoethanol, inhibit β-lapachone-induced apoptosis according to expectations. Camptothecin, another topoisomerase I inhibitor, also induces apoptosis, but HL-60 cells neither enhance H_2O_2 production, nor is camptothecin-induced apoptosis prevented by N-acetylcysteine (Chau et al., 1998). Obviously the anti-apoptotic effect of anti-

oxidants depends on H_2O_2-formation by the apoptogenic mechanism.

Hints to paradoxical effects of antioxidants came first from the observation that N-acetylcysteine induces apoptosis in vascular smooth muscle cells (Tsai et al., 1996), and has anti-tumour effects (Delneste et al., 1997). In recent years more evidence has accumulated for the idea that classical antioxidants stimulate radical formation, and have apoptotic properties. For example β-carotene, an accepted photoprotectant has even pro-carcinogenic activity (Black, 2004b).

In Jurkat cells (human T-cell leukaemia) incubation with 0.1–1 mM dehydroascorbic acid (that is rapidly taken up by cells and reduced to ascorbic acid) stimulated apoptosis of the cells that were exposed to H_2O_2. In contrast, preincubation with dehydroascorbic acid prevented apoptosis induction by camptothecin (Sane et al., 2004). Evidently ascorbic acid activates H_2O_2-induced apoptosis. However, although the prevention of camptothecin-induced apoptosis by ascorbic acid is not in agreement with the above-mentioned failure of N-acetylcysteine to antagonise camptothecin in a similar condition, it is nevertheless a hint to the complexity of antioxidant function.

The following observations illustrate the necessity to carefully explore antioxidants, before they may be considered for therapeutic use. Ascorbic acid at 0.25–1 mM causes a dose-dependent inhibition of leukaemia cell growth, and the oxidation of GSH, while ovarian cells were only minimally affected by the same treatment. The role of H_2O_2 production in apoptosis induction by ascorbic acid was further supported by the abrogation of programmed cell death in the presence of excessive catalase, and by the usual events associated with apoptosis induction (increase of Bax protein, release of cytochrome c, activation of caspase 9 and caspase 3, etc.) (Park et al., 2004).

Curcumin is an established antioxidant with anticancer properties. At low concentrations it diminishes ROS concentrations according to expectations: this effect is supported by water soluble antioxidants (ascorbic acid, N-acetylcysteine and GSH), but its anticancer properties rely on the promotion of ROS formation (Chen et al., 2005). Presumably a similar relationship exists for (−)-epigallocatechin gallate, another well known natural antioxidant. Nakagawa et al. (2004) reported that in Jurkat cells the Fe^{2+}-mediated formation of hydroxyl radicals ($HO^{\bullet}$) (Fig. 2) contributes importantly to cell death induced by this antioxidant.

A further complication of the mechanisms involved in the anticancer actions of antioxidants became recently apparent. Quercetin is known to inhibit cell proliferation. It had been claimed to have both anti-oxidant and pro-oxidant properties. Along with its anti-oxidant properties it prevents the apoptotic effect of H_2O_2 in HL-60 cells (Chen et al., 2004a). Its anti-proliferative effect is improved by ascorbic acid (1 mM), N-acetylcysteine (0.5 mM) or GSH (0.25 mM): the diminution of the cell proliferation rate was paralleled by a diminution of ROS formation (Chen et al., 2004b).

Recently the concept of α-tocopherol functioning as lipid-based radical chain breaking molecule has changed. A number of non-antioxidant functions are now attributed to vitamin E, such as stabilisation of mRNA and proteins, regulation of gene transcription and protein translation. These functions imply that vitamin E is protected by a network of cellular antioxidants, rather than being oxidised by ROS as other antioxidants are, which are present in tissues at high concentration (Azzi et al., 2003).

These few examples demonstrate that the antioxidants are two-faced, as Ianus, the Roman god: they may achieve protection from oxidative stress, inhibit cell proliferation, or enhance cell kill, depending on their concentration, the cell type (which implies the capacity to neutralise ROS) and the apoptogenic event.

Extracellular hydrogen peroxide formation

Since H_2O_2 is able to permeate the cell membrane, extracellular H_2O_2 induces signalling cascades even in the absence of a receptor ligand (DeYulia et al., 2005). Similarly extracellular H_2O_2 can exert toxic actions not only on the cell membrane, but also on intracellular structures. Owing to the absence of catalase in the cellular environment the half life of H_2O_2 outside cells is relatively long and signalling as well as toxic actions are favoured. A H_2O_2 generating reaction in the tumour environment was, therefore, considered to be a potential therapeutic target (Agostinelli et al., 1994a). In principle any H_2O_2 generating reaction could be taken into consideration for this purpose. Several examples of this idea have been published. To mention only a few:

H_2O_2 formed from by autoxidation of 6-hydroxydopamine caused cytolysis of neuroblastoma cells (Zaizen et al., 1986). The interaction of 1,3-propanediamine, putrescine and cadaverine with hog kidney diamine oxidase (DAO) arrested cell growth (Gaugas and Dewey, 1981), and immobilised DAO injected into the peritoneal cavity of mice inhibited the growth of Ehrlich ascites tumour cells (Mondovi et al., 1982). Microinjection into chick embryo fibroblasts and glioma cells demonstrated intracellular cytotoxicity of amine oxidases (Bachrach et al., 1987).

The fact that inhibitors of monoamine oxidase A (MAO A) protect against serum starvation-induced apoptosis (Malorni et al., 1998) indicates a role of MAOA in this model of programmed cell death.

a) Cytotoxicity of polyamine oxidation products

The so-called polyamines are a group of aliphatic biogenic amines. They attract interest because of their multiple vital functions in cell biology (Cohen, 1998), including among many others cell cycle regulation, gene expression and signal transduction (Bachrach et al., 2001; Childs et al., 2003).

The natural polyamines are formed from the decarboxylation products of ornithine and S-adenosyl-methionine in nearly all eukaryotic cells (for their structures see Fig. 4). Cellular polyamine concentrations are highly regulated. However in situations of over-accumulation or depletion of intracellular polyamine pools cell death may be induced (Schipper et al., 2001; Pignatti et al., 2004; Seiler and Raul, 2005).

Direct toxic effects are exerted only by high polyamine concentrations. Spermine, for example, is cytotoxic in the mM range. The cytotoxicity of spermidine and putrescine is even lower (Brunton et al., 1991; Seiler et al., 2000). Because they bind tightly to anionic sites of various cell constituents, the concentration of free polyamines is by far too low to be toxic, even though spermine and spermidine are present in some tissues at mM concentrations. For the same reason endogenous polyamines, in contrast with exogenous polyamines, do not exert unfavourable pharmacological effects (Seiler, 1991, 2005). Hence direct cytotoxic actions of the natural polyamines play no physiological role. However, several examples of cell death induction due to over-expression of ornithine decarboxylase are known: only in one case was cell death prevented in the presence of aminoguanidine (Erez et al., 2002), indicating that oxidative degradation of putrescine or spermidine was involved in the cytotoxic mechanism (aminoguanidine is a potent inhibitor of CuAOs (Nilsson, 1999).

The natural polyamines are substrates of several FAD-dependent enzymes (monoamine oxidase (MAO), PAO, spermine oxidase) and of CuAOs (diamine oxidase, serum amine oxidases). PAO is involved in the homeostatic regulation of polyamine pools. The other oxidases are important for the terminal catabolism of polyamines, i.e. they catalyse the formation of metabolites (ammonia, amino acids) that can be excreted via the kidneys (Seiler, 1992).

Products of polyamine oxidation, H_2O_2 and aldehydes (Fig. 4), are toxic to cells. In serum-containing cell cultures cytotoxicity of polyamines correlates with their property as substrates of serum amine oxidases (Morgan, 1988).

The products of polyamine oxidation have repeatedly been implicated in cytotoxicity (see e.g. Gaugas and Dewey, 1981; Agostinelli et al., 1994a; Calcabrini et al., 2002; Ha et al., 1997; Chen et al., 2001), inhibition of cell division (Bachrach et al., 1987; Henle et al., 1986) and apoptosis (Parchment, 1996). More recently it has convincingly been demonstrated that BSAO-catalysed spermine oxidation causes necrotic cell death of L1210 mouse leukaemia cells (Bonneau and Poulain, 2000): in fact death mechanisms induced by the products of polyamine oxidation are dependent of the cell type and of details of the experimental condition. Both non-apoptotic (necrotic) and apoptotic cell death due to exposure to spermine metabolites was observed in cultured cells and in vivo in a melanoma (Averill-Bates et al., 2005).

Both, aldehydes and H_2O_2 contribute to the cytocidal effect of polyamine metabolites. During short incubation times with BSAO and spermine H_2O_2 appears to be the major toxin, while the effect of the aldehydes is mainly observed after prolonged incubation. This is supported by the evaluation of the dose–response curves of exogenous H_2O_2 and acrolein, and by the following observation: if catalase or NAD-dependent aldehyde dehydrogenase (EC 1.2.1.5) were added individually to the culture medium in amounts sufficient to prevent accumulation to toxic levels of H_2O_2, respectively of aldehydes, cytotoxicity of H_2O_2 and of aldehydes, that were generated by spermine oxidation, was only partially diminished (Averill-Bates et al., 1993, 1994; Calcabrini et al., 2002). Cytotoxicity of spermine metabolites was, however, completely prevented in the presence of both, catalase and aldehyde dehydrogenase (Arancia et al., 2004).

The particular cytotoxic properties of acrolein (see Fig. 4) were emphasised in a more recent publication (Sharmin et al., 2001), and acrolein from spermidine and spermine was made responsible for the activation of genes of phase 2 enzymes, and of other genes that promote cell survival (Kwak et al., 2003). Polyamine-derived acrolein may be one of the uraemic toxins (Sakata et al., 2003). It has been shown that acrolein, which is also a metabolite of lipid peroxidation, is rapidly incorporated into proteins, generating protein-linked carbonyl derivatives. Their determination may be used as a measure of oxidative stress (Uchida et al., 1998). Polyamine-derived aldehydes other than acrolein may also modify protein structure, even

though they lack double bonds to undergo Michael addition (e.g. with thiols). In this regard it is interesting to notice that apoptosis is induced by some endogenous aldehydes, and that their accumulation may be related to age-related diseases (Davydov et al., 2004).

The protective role of the GSH/GSSG redox pair in the defence against excessive ROS has been discussed already (see first paragraph). As a rule, cells with a high GSH content are less sensitive to damage by spermine and spermidine metabolites, than cells with a low GSH content. It appears that GSH is not only important in the defence against BSAO-generated H_2O_2, but also against the aldehydes formed from spermine, as was shown for Chinese hamster ovary (CHO) cells, that were exposed to BSAO and spermine in the presence of excessive catalase (Agostinelli et al., 1996). While protective mechanisms by GSH have extensively been studied, protection against aldehydes is not as well understood. It has been postulated, that aldehydes form conjugates with GSH by a glutathione S-transferase-catalysed reaction (Agostinelli et al., 1996).

As was shown by Henle (1986) DNA damage in CHO cells parallels the concentration of spermidine in foetal bovine serum-containing culture medium. Under similar conditions (purified BSAO and spermine in the cell suspension medium) the pentose phosphate cycle (which provides deoxyribose for DNA synthesis) is activated, presumably as a compensatory protective reaction. By removing or adding glucose to the cell suspension medium in the presence of purified BSAO and spermine, a protective effect of glucose against H_2O_2-induced damage could be demonstrated (Averill-Bates and Przybytkowski, 1994; Agostinelli et al., 1996).

b) Bovine serum amine oxidase (BSAO)

The superfamily of amine oxidases (amine oxidoreductases) represent an important class of enzymes present in numerous living systems. Based on their cofactor, they are divided in two classes: FAD-containing, and Cu^{2+} and 2,3,5-trihydroxyphenylalanine quinone (TPQ) containing enzymes (Mondovi et al., 1989; James and Klinman, 1991).

Most of the work on ROS formation was performed using bovine serum amine oxidase (BSAO) (EC 1.4.3.6), the first enzyme found to metabolise preferentially spermidine and spermine (Hirsch, 1953). But other substrates of this enzyme (e.g. benzylamine, heptylamine and several other primary amines) are also known. The activities of CuAOs with specificity for spermidine and spermine are low in humans and most mammals. Except in rumi-

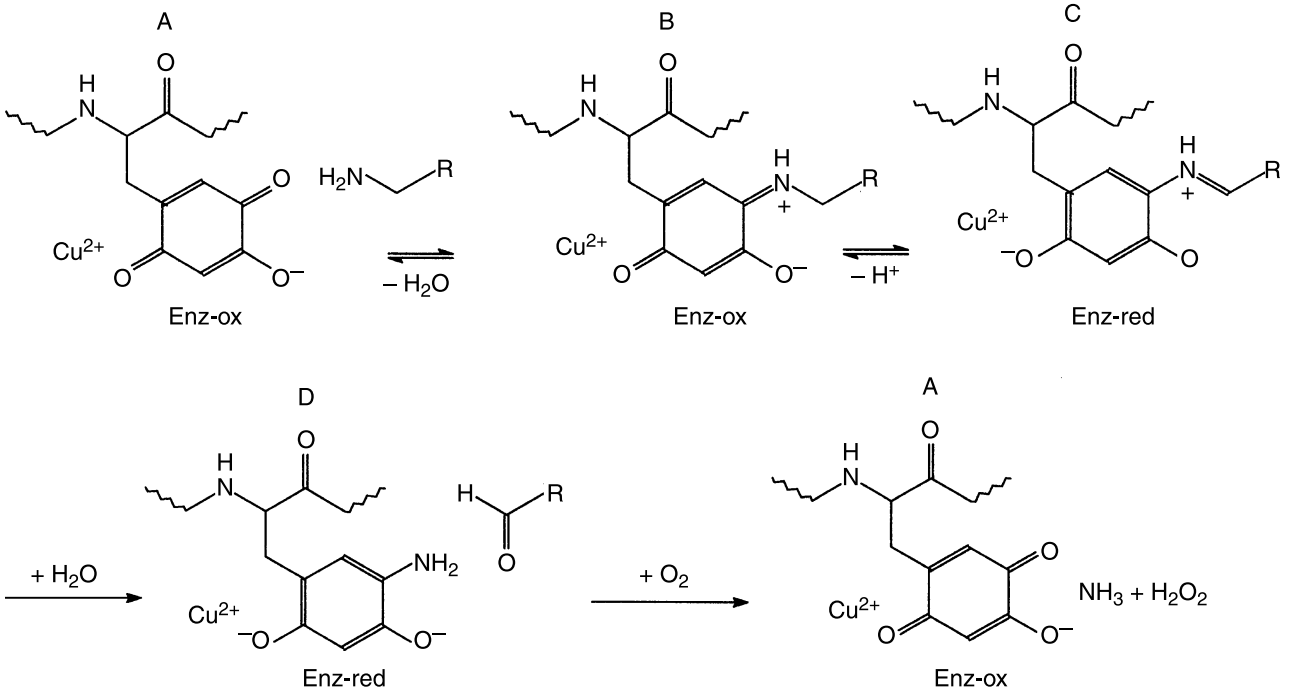

Fig. 3. Mode of action of the organic cofactor 2,3,5-triphenylalanine quinone (TPQ) of BSAO (according to Janes and Klinman, 1991). In the first step the amine forms a quinoneimine (B) with the oxidised form of the enzyme (A). Abstraction of a proton, and rearrangement of the double bonds analogous to that in transamination reactions forms the Schiff base (C), from which the aldehyde is released by hydrolysis. The oxidised form of the enzyme (A) is regenerated in the last step by reaction of 2 amino-3,5-dihydroxyphenylalanine (D) with O_2, and H_2O_2 and ammonia are released

nants they seem not to play a major role. The physiological function of BSAO is presumably the removal of excessive spermidine from the blood, spermidine that was liberated from bacteria. The oxidation products of spermidine are bactericide (Cohen, 1998).

BSAO is the prototype of mammalian CuAOs (for reviews see e.g. Houen, 1999; Agostinelli et al., 2004). All serum amine oxidases are dimers and have similar molecular organisations with 33 fully conserved residues close to the catalytic site. The peptide chains of the enzymes from different sources are sialylated to different degrees. Recently the crystal structure of desialylated BSAO has been reported at 2.37 Å resolution (Lunelli et al., 2005). Each of the identical subunit (M_r 85 kDa) contains in the active site one tightly bound Cu^{2+} coordinated to three His residues, and TPQ, which is part of the consensus sequence (Asn-TPQ-Asp (or Glu)) of the peptide chain (Mu et al., 1992).

The catalytic mechanism involves the formation of a Schiff base between the amine substrate and the oxidised TPQ that is followed by an intramolecular rearrangement of the double bonds. An aldehyde is released by a hydrolytic step. The cofactor in the reduced state, has at this stage an amino nitrogen covalently bound. Re-oxidation of TPQ by O_2 releases ammonia and H_2O_2 (Janes and Klinman, 1991) (Fig. 3). Cu^{2+} appears to play an important role in both the formation of the reduced enzyme species and its re-oxidation (Olson et al., 1978), and it appears to control the transfer of the substrate from a hydrophobic binding site near the protein surface to the deeply buried active site (Agostinelli et al., 1997; Parsons et al., 1995; Kumar et al., 1996). Cu^{2+} has also a structural function (Agostinelli et al., 1994b).

In the case of spermidine and spermine as substrates the primary amino group of the aminopropyl moiety is preferentially removed, to form the respective mono- and dialdehydes (Tabor et al., 1964; Lee and Sayre, 1998) (Fig. 4), but reaction of the secondary amino groups of spermine with purified BSAO has also been reported (not shown) (Houen, 1999). The aldehydes have been identified and studied for their biological properties (see e.g. Tabor et al., 1964). The formation of acrolein from these aldehydes by spontaneous β-elimination, was first postulated by Alarcon (1970), but has long been debated.

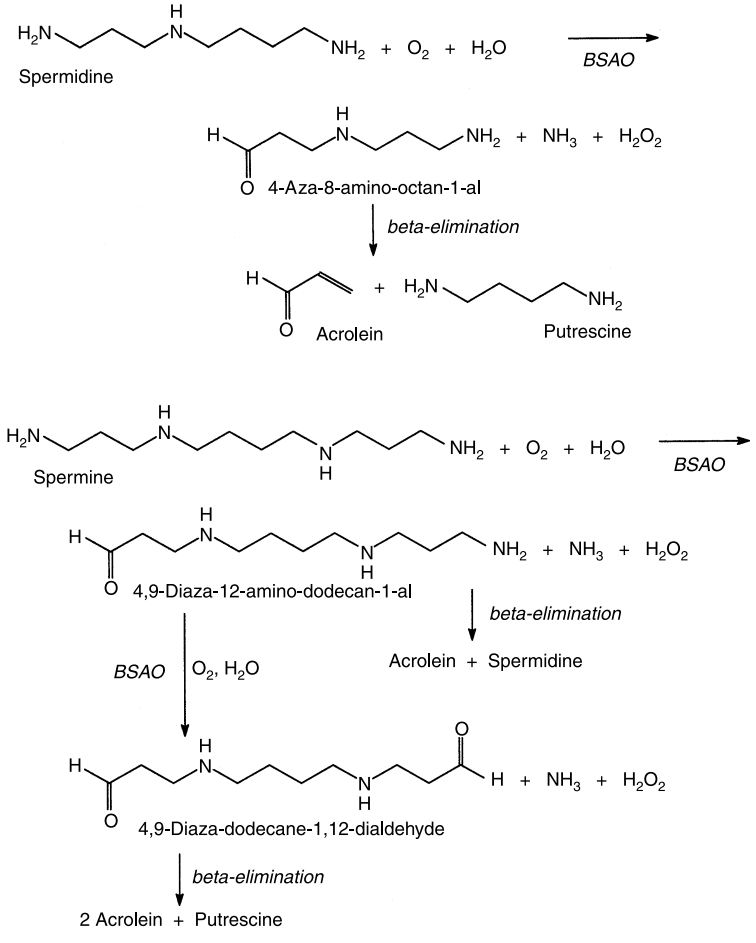

Fig. 4. Reaction scheme of the oxidative deamination of spermidine and spermine by bovine serum amine oxidase

It was particularly questioned by Israel et al. (1973), based on experiments with synthetic aldehydes. Recent work is in favour of the formation of acrolein as a product of BSAO-catalysed oxidation of spermidine and spermine in cell cultures, as has already been mentioned.

In the presence of suitable enzymes the aldehydes may be oxidised to the corresponding amino acids, or reduced to alcohols (not shown in Fig. 4; for details of polyamine catabolism see Seiler, 1992, 2004).

c) Death mechanisms induced by polyamine metabolites

Owing to the fact that mitochondria are not only the major source of ROS, but also the powerhouse of the cells, strategies were envisaged for the inhibition of mitochondrial respiration, with the aim to enhance drug-induced apoptosis by ROS (Pelicano et al., 2003). Metabolites of spermine oxidation have, as will be shown in the following, mitochondria toxic effects that may fit into the above strategy.

H_2O_2 is known to be able to cross the inner membrane of mitochondria and induces oxidative stress (Mathai and Sitaraman, 1994). As has been briefly discussed in the first paragraph, H_2O_2 is detoxified either by reaction with catalase, to form H_2O and O_2, or it is reduced by GSH or thioredoxine (Fig. 2). For the reduction of GSSG and oxidised thioreduxine NAD(P)H is required. The product of this reaction, $NADP^+$, is reduced by NADH (which is regulated by the respiratory chain and Bcl-2). This transhydrogenase-catalysed reaction leads to the mitochondrial uptake of H^+, and the membrane potential is affected. In the presence of high H_2O_2 concentrations $HO^\bullet$ is formed by Fe^{2+} catalysis (Fenton reaction) (Fig. 2). $HO^\bullet$ oxidises, among others, critical thiol groups of adenine translocase, which belongs to the mitochondrial permeability transition pore complex, and leads to pore opening. $HO^\bullet$ also stimulates membrane permeabilisation due to lipid peroxidation (Korshunov et al., 1997). Thus the redox state of mitochondria is impaired by ROS, initiating further mitochondrial damage.

Transmission electron microscopic examination of LoVo cells (which derive from a human colon adenocarcinoma) exhibited after exposure for 60 min to BSAO and spermine a generally well-preserved ultrastructure. Particularly the chromatin structure was not visibly affected. However, in agreement with the above discussed effects of H_2O_2 the mitochondria showed a condensed matrix, an altered cristae structure, and a slight swelling (Arancia et al., 2004).

Staining with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide (CJ-1) (Cossarizza et al., 1993) demonstrated a marked depolarisation of the mitochondrial membrane already 10 min after incubation with BSAO and spermine. Similar changes were observed after incubation with H_2O_2. These observations suggest the mitochondria as primary target of H_2O_2 that is generated in the cellular environment. The role of aldehyde toxicity in mitochondrial damage has not been adequately clarified, although some authors emphasized a prominent role of aldehydes in the induction of oxidative stress in the presence of phosphate (Kowaltowski et al., 1996).

The enhancement of intracellular ROS and a decrease of the GSH content of cells due to exposure to BSAO and spermine was also observed. All the above mentioned changes were more pronounced in drug resistant LoVo cells than in the corresponding wild type cells, in agreement with the greater sensitivity of the former to damage by polyamine metabolites (Arancia et al., 2004). One among several possible interpretations of these findings is that differences in structural and/or functional properties exist between the mitochondria of multidrug resistant cells and the corresponding wild type cells. Along this line of thinking it was hypothesised (Jia et al., 1996) that multidrug resistant cells have an increased activity of the mitochondrial electron transport chain, because they highly express ATP-dependent P-glycoprotein (Jia et al., 1999). A part of the mitochondrial ROS derives from the electron transport chain. The cellular defence system can normally cope with physiological ROS production (Kowaltowski and Vercesi, 1999), but not with a high rate of ROS formation. Since treatment with BSAO and spermine causes a higher increase of the ROS level in multidrug resistant LoVo cells than in the corresponding wild type cells, a higher grade of mitochondrial damage is the consequence.

Perspectives of clinical applications

Catalytically liberated cytotoxic agents have the advantage that only a small amount of the enzyme is required for toxin formation, and that the cytotoxic reaction products are continuously formed over an extended period of time. In the case of BSAO the substrates, spermidine and spermine, are generated by the tumour cells themselves, and they are released into the cellular environment, if cells are damaged. Thus, if BSAO is placed in the immediate vicinity of tumour cells, the toxic products, H_2O_2, and aldehydes will be continuously produced, and more and more substrate will become available to the enzyme due to cell kill. In other words, a positive feedback mechanism enhances the efficacy of BSAO catalysed cell damage (Fig. 5). Since the products of spermine oxidation are

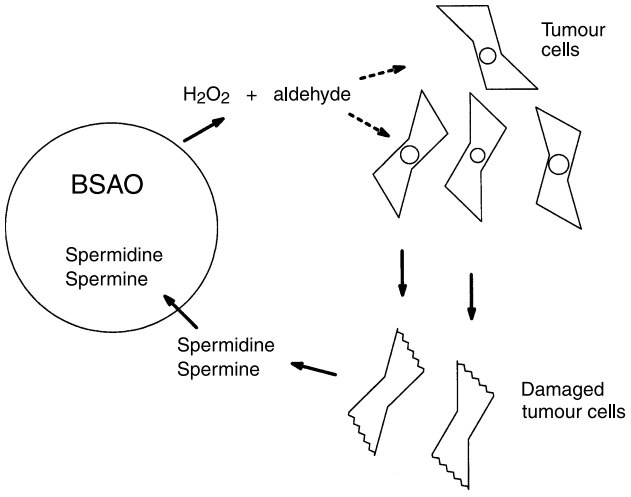

Fig. 5. Scheme illustrating the positive feedback effect of cell damage by bovine serum amine oxidase (BSAO) that was located within or near a tumour. The substrates of this enzyme, spermidine and spermine, are released from tumour cells spontaneously and from damaged cells, and are transformed into cytotoxic products in the vicinity of tumour cells, so that they induce cell death

chemically reactive, and H_2O_2 has in addition a limited life span, the cytotoxic metabolites remain located in a small area near the site of their formation. An advantage of this approach is also the possibility to stop the formation of cytotoxic metabolites by administration of non-toxic doses of aminoguanidine.

Although only a few micrograms of enzyme protein are required for inhibition of tumour growth, attempts were made to incorporate the enzyme into liposomal vesicles (Agostinelli et al., 1988), and to prepare complexes with gold that are bound and incorporated by hepatocytes (Dini et al., 1991). Furthermore BSAO was incorporated into biocompatible, non-immunogenic polyethylene glycol hydrogels (Demers et al., 2001). The immobilised enzyme exhibited considerable advantages over the free enzyme: thus, for example, the growth of a mouse melanoma (B16-F0) was reduced by 70 percent after a single injection of the immobilised enzyme, in comparison with 32 percent inhibition after injection of the same amount of native enzyme. While the immobilised enzyme induced a high level (70 percent) of apoptosis, non-apoptotic cell death prevailed in the case of native enzyme (Averill-Bates et al., 2005). The difference was attributed to the slow, gradual release of spermine oxidation products from the hydrogel, i.e. the long-term exposure of the tumour to ROS and aldehydes, as compared with the shorter, though more rapid release of toxic metabolites by the native enzyme.

Based on the above-discussed release of the substrates of ROS formation from tumour cells, adjunct treatments suitable to enhance cell death, or impair tumour cell growth by mechanisms which synergise the effect of ROS are of obvious advantage. Combinations with conventional and new anti-tumour drugs are currently investigated.

Since hyperthermia is a clinically established therapeutic method, strategies should be developed that combine hyperthermia with extracellular ROS formation. In support of this idea is the fact that a marked enhancement of cytotoxicity was observed by elevating the temperature of tumour cell cultures from 37 to 42 °C. Under these conditions the spermine-derived aldehydes were mainly responsible for the increased rate of cell death (Agostinelli et al., 1994a). An additional advantage derives from the fact that an increase of the incubation temperature, in the presence of BSAO and spermine, increases the proportion of P-glycoprotein over-expressing multidrug resistant CHO cells (Lord-Fontaine et al., 2001). Since, as has already been discussed, multidrug resistant cells are more sensitive to toxic polyamine metabolites than the wild type cells (Arancia et al., 2004), hyperthermia supports apoptosis by several mechanisms.

A potential disadvantage of polyamine oxidation is the impairment of inerleukin-2 production by human peripheral blood mononuclear cells, the consequent inhibition of T-cell proliferation, and the impairment of the immune defence system (Flescher et al., 1989). To what extent this sequence is valid under therapeutic conditions has not yet been investigated.

Conclusions

In contrast with therapeutic methods implying irradiation-derived radicals, drug-induced radical formation is simple and cost effective, because these methods are not dependent of sophisticated, expensive devices. However, therapeutic applications of radical generating systems are, with few exceptions, still in their infancy. They may gain importance, if major problems have been solved. Among these the targeting of tumours by anticancer drugs is one of the most difficult problems. For the time being, tumours which are surgically accessible should be the main targets for enzyme-catalysed radical formation. A major advantage of this method is the continuous formation of cytotoxic products over an extended period of time within the tumour, or in its vicinity. ROS formation may become particularly useful in assisting to overcome a major problem of conventional anticancer therapy, namely the development of drug resistance, because it has been demonstrated that multidrug resistant cells are more sensitive to the treatment with BSAO and spermine than their wild type counterparts.

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