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Cigarette smoke and the involvement of free radical reactions in chemical carcinogenesis

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Cancer is a multi-step process, involving at least two stages that can be experimentally distinguished: initiation and promotion. Initiation involves an irreversible alteration of the cellular DNA that permits the carcinogenic transformation of the cell. Promotion produces conditions that allow the initiated cell to become clonally unstable so that it actually does produce a tumour. Promotion generally occurs over a fairly long period and is reversible.

Promotion involves a complex cascade of cellular changes, not all of which are understood. However, superoxide and other oxy-radicals are known to be involved in promotion, a process that is essential for a tumour to develop (Cerutti, 1985; Slaga *et al.*, 1981). Since promotion is reversible, there continues to be hope that the use of antioxidant and other strategies that control free radical reactions can protect initiated cells against promotion and, thus, prevent the ultimate development of a tumour (Shamberger *et al.*, 1973; Shankel *et al.*, 1986).

The involvement of radicals in promotion has been reviewed by a number of authors (Heckler *et al.*, 1982; Slaga, 1983; Slaga *et al.*, 1981; Smith *et al.*, 1983; Troll *et al.*, 1982). In this article, I wish to turn instead to a more controversial and even less understood area, the involvement of free radiclas in the initiation of tumour development.

Our thoughts on the involvement of free radicals in cancer have stemmed from our research on cigarette smoke, and I will briefly review some of those results. We have reported that cigarette tar contains a stable, long-lived free radical that is a semiquinone embedded in a low molecular-weight, tarry matrix (Church & Pryor, 1985; Pryor *et al.*, 1983). This tar radical has a stable electron spin resonance (ESR) signal, and when the tar radical and DNA are incubated together, an ESR signal appears in the reisolated DNA (Pryor *et al.*, 1984). When we reported this finding, chemists familiar with the classical literature on chemical carcinogens immediately began asking questions such as: Which component of tar binds to DNA? To which base does the binding occur, and is the binding covalent?

Questions of this type have led to very profitable research in the study of the chemical carcinogenesis of polynuclear aromatic hydrocarbons (PAH) (Harvey, 1985). For example, the discovery that benzo[a]pyrene (BaP) is activated by the cytochrome P450 system to a dihydrodiolepoxide (BPDE), and that this species then binds to DNA in a stereospecific way, has allowed the study of chemical carcinogenesis on a molecular level in precise and elegant ways (Thakker *et al.*, 1985).

Thus it was quite natural for us to ask questions such as: Which species in tar binds to DNA? However, we now believe that this may be the wrong question to ask with regard to damage in DNA caused by large free radicals.

Before discussing why probing the binding of carcinogens to DNA may not be the correct approach when radicals are involved, let us consider in more detail the implications of the ESR experiment described above (Pryor *et al.*, 1984). The association of the tar ESR signal with DNA does not *necessarily* mean that a component of tar has bound to DNA. A priori, there are three possibilities for interaction of the tar radical with DNA that seem ressonable. The first is for the free radical centre in the tar to attack a DNA base and add to it; this would produce a radical centre on the DNA that would then be expected to pick up a hydrogen atom. This sequence leads to a stable adduct of the tar radical with DNA that no longer is a free radical and would *not* give an ESR signal. The second possibility would be for the tar radical to bind to DNA through its hydroquinone or quinone functionalities. If this were to occur, the tar free radical centre would simply by carried along and the adduct would retain its semiquinone ESR signal largely unchanged. The third possibility would be for the tar radical to bind to DNA by some reaction, perhaps losing its radical centre in the process, and the tar hydroquinone to be reoxidized to a radical centre while bound to DNA. Our data make it appear unlikely that an *unmodified* tar radical has bound to DNA, since the g-value of the ESR signal increases slightly when the signal becomes associated with DNA. Thus, *none* of these possibilities for the interaction of the tar radical with DNA appear to fit our observations. Therefore, we are currently entertaining the possibility that the tar radical does not bind to DNA. [If this were true, the DNA ESR signal may result from oxy-radical damage to DNA; see below.]

If the tar radical does *not* bind to DNA, what other possibility exists for tar to produce DNA damage? It is well known that many quinones undergo what is called redox cycling (Ts'o *et al.*, 1977). In this process, the quinone is reduced or the hydroquinone is oxidized to a semiquinone, which in turn reduces oxygen to produce superoxide. Superoxide then leads to the production of hydrogen peroxide, either spontaneously or catalyzed by superoxide dismutase. Hydrogen peroxide is decomposed by transition metals such as iron or copper to form the hydroxyl radical (Aust & Svingen, 1982). It is well known that the hydroxyl radical produced by radiation or by chemical reactions is an extremely reactive species that attacks DNA to produce a large number of modified bases, strand breaks, and other types of DNA damage (Ames *et al.*, 1984; Cavalieri & Rogan, 1984; Lesko *et al.*, 1980; Birnboim, 1982; Cerutti, 1985).

Shortly after discovering that the ESR signal becomes associated with DNA when tar and DNA are incubated together, we found that the tar semiquinone radical could be partially extracted into aqueous buffers (Cosgrove *et al.*, 1985). We also found that these buffered solutions reduce oxygen to superoxide, and, since they also contain metals, reduce hydrogen peroxide to produce the hydroxyl radical. We were able to both spin trap hydroxyl radicals from aqueous extracts of cigarette tar and show that these extracts cause DNA nicks (Borish *et al.*, 1985). Furthermore, scavengers that block the production of the hydroxyl radical spin adduct also block the production of DNA nicks, indicating that both arise from hydroxyl radicals (Borish *et al.*, 1986). Thus, our research turned from asking questions about the species in tar that *bind* to DNA to asking questions relevant to the production of superoxide, the subsequent formation of the hydroxyl radical, and the interaction of the hydroxyl radical with DNA.

We now believe that the cigarette tar radical possesses five properties that make it especially likely to damage DNA, and that all five of these are critical. Firstly, the semiquinone tar radical is long lived. Secondly, it is partially water

soluble. Thirdly, the tar radical associates with DNA. Fourthly, the tar semiquinone functionality reduces dioxygen to form the superoxide radical, which then produces hydrogen peroxide. And lastly, tar (perhaps through its phenolic functionalities) chelates metal ions such as iron or copper. Figure 1 is a cartoon showing some of these functions. The association of tar with DNA is an essential part of the damage-producing process. This is true because the hydroxyl radical is an extremely short-lived species, with a halflife of only 10^{-9} sec, which can only diffuse 10–30 molecular diameters before it reacts (Pryor, 1986*a*). If the hydroxyl radical is not formed very near the DNA, it will react with another species before it reaches the DNA.

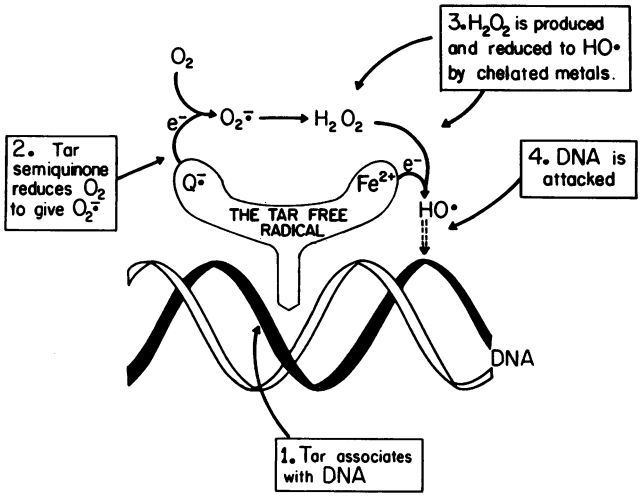

Figure 1 DNA-damaging properties of the cigarette tar radical.

All of this led us to think in a general way about mechanisms by which radicals could be involved in the production of cancer. Table I summarizes possible mechanisms in which radicals might be involved in causing DNA damage. As can be seen, I have divided the possible mechanisms into five classes based on which steps, if any, are radical mediated. Class I is the example that has become familiar from the studies of BaP activated by P450. Most workers believe that P450 catalyzes the oxidation of BaP to the dihydrodiol-epoxide by reactions that do not involve *free* radicals (Harvey, 1985). [Radicaloid species may be involved however (Ortiz de Montellano *et al.*, 1983).] Thus, the entry in the first column in Table I is 'No', since the activation of BaP by P450 does not appear to involve free radical intermediates. Furthermore, the BPDE intermediate binds to DNA by a mechanism that involves electrophilic centres in DNA attacking the epoxide functionality. Thus, binding of this carcinogen also does not involve free radicals, so mechanism I also gets a 'No' in column 2 of the Table.

Mechanism II also is a fairly well established pathway. One of the best-known examples is the oxidation of the diol of BaP to the BPDE intermediate by radicals produced during the activity of the prostaglandin synthetase enzyme (PGS) system (Marnett, 1984). Thus, the same BPDE intermediate is produced from BaP by this route as is by the P450 route, but in this case the PGS enzyme system is responsible and radicals are involved in the activation step. Since the same dihydrodiolepoxide intermediate is produced, it again binds to DNA by a process that does not involve radicals. Thus, mechanism II has a 'Yes' in column 1 and a 'No' in column 2 in Table I.

Another possibility for mechanism II is the oxidation of a PAH to hydroquinones and quinones. The oxidation of the PAH could involve radical reactions (Ts'o *et al.*, 1977), but the binding of the quinone or the hydroquinone to DNA could involve electrophilic reactions. Thus, the activation of the procarcinogen could be radical but the binding non-radical, as shown in mechanism II.

Mechanisms III and IV, which involve the binding of a radical species to DNA, are both quite rare. In fact, there does not appear to be an unequivocal example of a large organic radical binding to DNA by a free radical mechanism. Reactive radicals certainly would be expected to bind to DNA if they were produced in its vicinity. It is intriguing that the binding of species to DNA via ionic reactions has been characterized in such great detail but no examples of the addition of large radicals have been worked out. Either radical binding is more difficult to detect with the carcinogens that have been studied to date, or large fragments do not bind to DNA by radical pathways, despite the ability of radicals to add to double bonds and to rings. The hydroxyl radical *does* add to DNA bases, and apparently does so very radily *in vivo* (Ames *et al.*, 1984; Demple & Halbrook, 1983); however HO• is much more reactive than is a large organic radical. Furthermore, I believe that association of species (like tar) with DNA preceeds the production of the HO• radical, which is then produced in the immediate vicinity of DNA. Perhaps large radicals are not usually produced *close enough* to DNA, and they become inactivated as they diffuse toward DNA.

One example of a large free radical binding to DNA is interesting in this context. Mitomycin C can be activated to a semiquinone by a one-electron process or to a quinone by a two-electron process, and the mitomycin C semiquinone may bind to DNA by a free radical mechanism (Kennedy *et al.*, 1985). However, even if it does, it is an event that often leads to cellular death.

A further remark should be made about mechanisms III and IV. Some quinones can be reduced to the hydroquinone by DT-diaphorase enzymes in a two-electron process that does not involve free radicals. The resulting hydroquinone, however, can spontaneously oxidize to the semiquinone. Thus, although the enzymatic conversion of the quinone to the hydroquinone may not involve radicals, semiquinone radicals may be formed, and these *could* bind to DNA by a

Table I Possible roles of free radical reactions in chemical carcinogenesis

Mechanism number	*Are radical reactions involved in the* activation *of the procarcinogen to its carcinogenic form?*	*Are radicals involved in the* binding *of the carcinogen to DNA?*	*Possible examples (see text)*
I	No	No	BaP/oxidation by P450
II	Yes	No	BaP/oxidation by PGS
III	No	Yes	?
IV	Yes	Yes	?
V	Oxy radicals produce DNA damage (e.g., thymine glycol) but *binding* of the carcinogen does not occur.		Radiation, bleomycin, cigarette tar

radical pathway, in theory at least. This, therefore, is a mechanism with some features of type III, although, as we will discuss below, most semiquinones probably do not *bind* to DNA but instead reduce oxygen to superoxide.

Despite these possibilities for mechanisms III and IV to rationalize radical involvement in chemical carcinogenesis, it appears that mechanism V, the production of oxy-radicals, is the most common way in which free radicals produce damage to DNA. Ionizing radiation, bleomycin, adriamycin, and cigarette tar all produce superoxide and a cascade of activated oxygen species that ultimately lead to the hydroxyl radical. The hydroxyl radical then is able to both abstract hydrogen atoms from DNA (e.g. converting thymine to hydroxymethyluracil) and add to DNA (e.g. producing thymine glycol). Thus, unlike electrophilic processes, chemical carcinogenesis by free radicals may *not* involve the binding of a *large fragment* from the carcinogen to DNA.

Mechanism V, which involves radicals but not binding of the carcinogen to DNA, is illustrated in Figure 2 for the case of a PAH (or similar species) that can be oxidized to hydroquinone (QH_2) and quinone (Q) species. In eq 1, the substrate is ezymatically oxidized to a hydroquinone by a process that can be multistep; the oxidation of BaP by either PES or P450 is an example of such a conversion. The quinone then can be reduced to the hydroquinone, for example by DT-diaphorases, eq 2. Alternatively, the hydroquinone can undergo spontaneous oxidation to the semiquinone, $QH^{\bullet}$, eq 3. The quinone also can be converted directly to the semiquinone, for example, by accepting an electron from a reductase, as shown in eq 4. The semiquinone then initiates the well-established processes leading to the damaging hydroxyl radical, eqs 5–7. The result of these reactions is that a procarcinogen is activated, either by radical or non-radical reactions, to species that produce oxy-radicals. These oxy-radicals initiate DNA damage that could lead to cancer, but the activated form of the carcinogen does not bind to DNA.

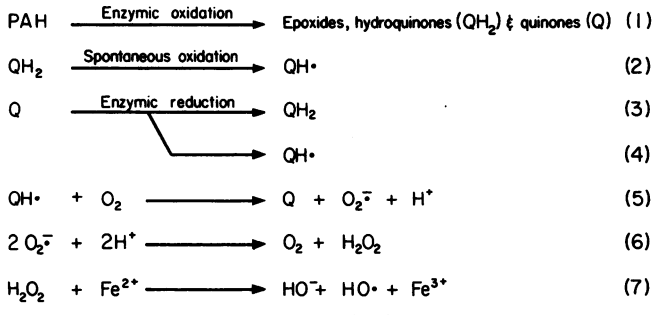

Figure 2 Mechanism V.

There is an indirect way in which radicals could be involved in carcinogenesis. Since oxidative stress is so common for the cell, and since radical reactions undoubtedly damage *all* biopolymer molecules in the cell, radical damage must inevitably lead to cell death in some cases. The death of cells produces a cellular environment of new synthesis that makes the cell particularly susceptible to carcinogenic transformation.

Cells appear to be bathed in a continuous flux of oxy-radicals, produced by the oxidative stress that is commonplace to aerobically metabolizing cells. Oxygenated derivatives of DNA bases are continually excreted by animals at extremely high levels (Ames *et al.*, 1984). Thus, radical damage to DNA is an ongoing fact of life that the cell must deal with (Cavalieri & Rogan, 1985; Cutler, 1984; Pryor, 1976, 1977, 1978, 1982, 1984, 1986*a*, *b*). Therefore, it may be critically important that radicals do not generally lead to the binding of large adducts to DNA, but instead produce a rather limited number of modified bases (such as thymine glycol) that can be enzymatically repaired in reasonably error-free processes (Demple & Halbrook, 1983; Demple & Linn, 1982).

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Discussion

Adams: These reactions with DNA have been studied a great deal by radiation chemists and the major reaction by far is the addition to the double bond rather than hydrogen atom abstraction from the methyl group. Yet Ames finds that the hydroxyl methyl derivative is the major one in the urine.

Pryor: Differing effects on yield due to the enzymology of the repair of these different types of damage could be responsible for this.

Adams: We always end up with a hydroxylated base.

Pryor: Bruce Ames points out that these lesions must occur *in vivo* because there are specific glycosylases to handle them. It is not necessary, however, that the efficiency of recovery of hydroxymethyluracil and thymine glycol in the urine be identical if equal amounts of both were produced.

Alper: Am I right in thinking that you are assuming that when Ames sees this product that this is an indication that the cells have been destroyed or killed by $OH^{\cdot}$ attack?

Pryor: I believe that Bruce Ames does not assume that, if I can speak for him. I believe he thinks that the repair is so ongoing, so continuous and so important that cell death does not result, although these products are being cleaved out and excreted and transported from the cell into the urine.

Alper: Couldn't there be another possibility. Since every animal sheds millions of cells every day, it could very well be that these products are made after the cells have died and it is really nothing to do with what happens inside the cell.

Pryor: Well I don't know where Bruce Ames is when I need him but he has published quite extensively on this now and I believe he has eliminated that possibility on control experiments.

Ward: It would be of interest to search for these products in human patients who have been subjected to whole body irradiation.

Pryor: I agree. One of the things that needs to be done is to study the effect of oxidative stress. Ames has done some of this and the initial results are disappointing.

Dean: Are there analogous studies on anaerobic bacteria, whether irradiated or not? Do anaerobic bacteria produce these products when they are irradiated?

Pryor: I would think not. Isn't oxygen required to form hydroxymethyluracil?

Dizdaroglu: Hydroxymethyluracil (HMU) can also be formed without oxygen; the $CH_2^{\cdot}$ is oxidised somehow. It either reacts with OH^- or adds oxygen.

Forni: Does the tar radical show any evidence of associating with a particular DNA site?

Pryor: We did not do that. We stopped research of that type when we learned that tar reduces oxygen to produce reactive oxygen species. To do that experiment it would be nice to have the radioactive tar radical, and we think we can make it. But I must first emphasise again we do not have one species. When we do HPLC on tar we get a mess.

Forni: The other thing is, do you think that your tar radical can directly cause cell death? Because in other studies endothelial cells die instantly when exposed to smoke.

Pryor: Cigarette smoke is an oxidative threat to cells. For example, we have shown that the protein a1PI is inactivated by smoke, and I am sure that also is true of other proteins. [Pryor *et al.* (1986), *Adv. Free Rad. Biol. Med.*, **2**, 161].