Exposure to Cigarette Tar Inhibits Ribonucleotide Reductase and Blocks Lymphocyte Proliferation

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Cigarette smoking causes profound suppression of pulmonary T cell responses, which has been associated with increased susceptibility to respiratory tract infections and decreased tumor surveillance. Exposure of human T cells to cigarette tar or its major phenolic components, hydroquinone and catechol, causes an immediate cessation of DNA synthesis without cytotoxicity. However, little is known of the mechanisms by which this phenomenon occurs. In this report we demonstrate that hydroquinone and catechol inhibit lymphocyte proliferation by quenching the essential tyrosyl radical in the M2 subunit of ribonucleotide reductase. The Journal of Immunology, 2000, 165: 6771–6775.

Ribonucleotide reductase is an iron-dependent enzyme that catalyzes the reduction of ribonucleotides to deoxyribonucleotides. Eukaryotic ribonucleotide reductase consists of two homodimeric proteins, M1 and M2, which combine to form the catalytic site. The M1 subunit contains the sulfhydryl electron donors for reduction of the ribose moiety, while M2 protein has a stable tyrosyl radical that initiates M1 redox reactions (11, 12). Both the tyrosyl radical and its stabilizing dfferic iron center are essential for enzyme activity. Quenching of the radical by hydroxuryla inactivates the enzyme, and removal of intracellular iron by chelators results in a disappearance of the radical (13–15). The experiments presented here were designed to test the hypothesis that the phenolic components of cigarette tar, HQ and catechol, block lymphocyte proliferation either by transferring an electron to the tyrosyl radical (quenching) or by chelating iron needed to maintain the tyrosyl radical.

Materials and Methods

Reagents

All chemicals were purchased from Sigma (St. Louis, MO) and dissolved in sterile PBS. The structures and reduction potentials of the phenolic chemicals used are illustrated in Fig. 1. Cigarette tar extracts were prepared by “smoking” a single cigarette into 10 ml RPMI 1640 via a vacuum pump at 125 ml/min. In these studies low- and high-tar cigarettes were represented by filtered Carlton (Brown and Williamson Tobacco, Louisville, KY; 0.1 mg tar and 1 mg nicotine per cigarette) and unfiltered Camel (R.J. Reynolds Tobacco, Winston-Salem, NC; 26 mg tar and 1.7 mg nicotine per cigarette), respectively.

Cell culture and analysis of DNA synthesis

Jurkat T cells were cultured in complete medium, which consisted of RPMI 1640 (Life Technologies, Rockville, MD) supplemented with 10% FBS (Mediatech, Herndon, VA), 50 U/ml penicillin, 50 μg/ml streptomycin, and 25 μg/ml gentamicin. To ensure that cells were in exponential growth, media were changed 24 h before all experiments. Cells were grown to a final density of 1.5 × 10⁶ cells/ml. DNA synthesis was measured by culturing cells in 96-well plates with or without various agents for 1 h, then pulsed with 1 μCi/well [3H]TdR for 2 h. Cells were harvested onto glass fiber filters using a cell harvester (Harvester 96; Tomtec, Orange, CT) and radioactivity was quantitated by liquid scintillation spectroscopy.

Quantification of phenolic compounds in cigarette smoke extracts

Cigarette tar extracts were prepared as above and filtered through 0.45 μm filters. Samples (100 μl) were analyzed by reverse-phase C18 HPLC using a 4.6 × 150 mm symmetry C18 column (Waters, Milford, MA) and a 4.6 × 12.5 mm Eclipse XDB-C18 guard column (Hewlett Packard, Palo Alto, CA) monitored by an electrochemical detector (7 mV) (16). Peaks

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—3 Abbreviations used in this paper: HQ; hydroquinone; HTL, human T lymphocytes; EPR, electron paramagnetic resonance; K, Kelvin.

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representing HQ and catechol were identified at 6 min and 13 min, respectively, and the area under the curve was quantitated using HQ and catechol standards and Millennium software (Waters).

Electron paramagnetic resonance (EPR) analysis of packed cell pellets
Jurkat T cells (3 \times 10^6) were harvested and packed by centrifugation for 5 min at 500 \times g in 4 mm (outside diameter) quartz EPR tubes, which were subsequently frozen and stored in liquid nitrogen. The overall time taken for spinning and freezing was less than 20 min. EPR spectra were recorded at 93° K using a Bruker E580 spectrometer with microwave power of 28 mW and a microwave frequency of 9.56 GHz. The absolute tyrosyl radical concentration in untreated Jurkat cells was determined at 93° K by comparison with a 1 mM stable nitroxyl radical \(\text{M}^\cdot\text{O}_{\text{g}}\) standard. For tyrosyl radicals and catechol:iron chelates, the characteristic g value was determined according to the formula \(g = h \beta / B_1\), where \(h\) is Planck’s constant, \(\beta\) is the microwave power, and \(B_1\) is the magnetic field.

Results
Inhibition of DNA synthesis by phenolic components of cigarette tar
Previous studies implicating inhibition of ribonucleotide reductase by HQ and catechol used both HTL and transformed lymphocytes. For these studies, the Jurkat T cell line was selected to accommodate the requirement for greater than 10^8 rapidly dividing cells for each EPR sample, because only cells expressing very high levels of M2 subunit display detectable EPR signals. Thus, to demonstrate the antiproliferative effects of cigarette tar and its phenolic constituents on Jurkat T cells, [3H]thymidine uptake was measured in cells treated for 1 h with cigarette tar, HQ, or catechol. Water-soluble extracts were prepared by smoking a single cigarette into 10 ml RPMI 1640 via a vacuum pump at 125 ml/min. High-tar extracts (unfiltered Camel) were more potent inhibitors of DNA synthesis than low-tar extracts. At a dose of 1 cigarette/20 ml (1.5 \times 10^6 cells/ml), the low-tar extract (filtered Carlton) inhibited DNA synthesis by greater than 85% (4,966 \pm 827 cpm) relative to untreated cells (37,707 \pm 675 cpm). However, high tar extracts inhibited thymidine incorporation to a similar degree at doses as low as 1 cigarette/100 ml (4,377 \pm 814 cpm). HQ and catechol also inhibited DNA synthesis in a dose-dependent manner. At 40 \mu M, HQ or catechol inhibited [3H]thymidine uptake by 70–80% (11,067 \pm 665 cpm and 6,750 \pm 264 cpm, respectively). In contrast, 40 \mu M phenol and 1 mM nicotine had no effect on DNA synthesis.

Detection of M2 tyrosyl radical and effects of cigarette tar extracts
To identify the effects of cigarette tar on the M2 subunit, we analyzed the EPR signal of the tyrosyl radical in Jurkat T cells. EPR detects the absorption of electromagnetic radiation by unpaired electrons, such as the tyrosyl radical present in the M2 subunit. The amount of tyrosyl radical, which is directly proportional to ribonucleotide reductase activity, was determined from the peak height of the characteristic g = 2.005 EPR signal. This EPR signal has been measured previously in frozen cell pellets of a variety of mammalian cell lines (17–19). Packed Jurkat T cells exhibited a g = 2.005 EPR spectrum at 50° K similar to the previously reported tyrosyl signals of other human leukemic cell lines (14, 15). The tyrosyl signal was also detectable at 93° K with equal reliability with an absolute intensity 0.2 \mu M. (Fig. 2). Thus, representative samples were analyzed at 50° K, but the loss of the tyrosyl radical was measured predominantly at 93° K.

The tyrosyl EPR signal was measured in Jurkat T cells exposed to water-soluble cigarette smoke extracts at a dose of 1 cigarette/10 ml cell culture. During a 5-min incubation at room temperature, high-tar extracts completely quenched the signal at g = 2.005, whereas <50% of the tyrosyl radical was quenched after treatment with low-tar extracts (Fig. 2). Although other effects on T cells cannot be excluded, this effect is consistent with the direct correlation between tar content and immunosuppression previously reported (7).

The concentrations of HQ and catechol in the cigarette smoke extracts were determined by reverse-phase HPLC monitored by an electrochemical detector (7 mV) in series with a UV-detector (16). High-tar extracts contained 12 \mu M HQ and 14 \mu M catechol, while the levels of HQ and catechol in low-tar extracts were below detection limits (1 \mu M) of this system. These results indicate that the dose of cigarette extract that quenches the tyrosyl radical contains levels of HQ and catechol that are known to inhibit DNA synthesis (8–10).

Effects of phenolic compounds on M2 protein tyrosyl radical
The effects of the phenolic tar constituents on the tyrosyl radical of M2 protein were determined by treating Jurkat T cells with HQ, catechol, or phenol for 5 min and then freezing the cells in liquid nitrogen.
The effect of nicotine, another putative immunomodulatory component of cigarette smoke, was also determined. Complete disappearance of the tyrosyl radical was observed in Jurkat T cells treated with 40 μM HQ or catechol (Fig. 3) and was identical with the effect of the known quenching agent, hydroxyurea (data not shown). Phenol (40 μM) had only a modest effect on the amplitude of the tyrosyl radical signal, and 1 mM nicotine had no effect (Fig. 3). These results suggest that inhibition of ribonucleotide reductase by HQ and catechol involves direct transfer of a reducing equivalent (electron or hydrogen radical) to a preformed tyrosyl radical in the M2 subunit. This proposed mechanism is supported by the direct correlation between the reduction potentials of HQ, catechol, and phenol (459 mV, 530 mV, 800 mV, respectively; Ref. 20) and their effects on the tyrosyl radical.

HQ and catechol exhibited nearly identical dose-dependent effects on the tyrosyl radical (Fig. 4), but did not cause permanent inactivation of M2 protein. Reappearance of 60–80% of the tyrosyl radical was observed in Jurkat T cells treated for 5 min with 40 μM catechol and HQ, respectively, and subsequently washed in the presence of cycloheximide to prevent de novo M2 protein synthesis (Fig. 4). Although protein synthesis was not measured directly in these studies, less than 30% of M2 protein normally turns over per hour (21), indicating that de novo synthesis alone cannot explain the level of reappearance of radical seen in washed cells.

**Detection of iron-chelate complexes**

In addition to being a reducing agent, catechol functions as an iron chelator (22). The inhibitory effects of other iron chelators have been attributed to a passive removal of iron from the medium, which prevents regeneration of the iron-radical center, rather than to an active removal of iron from the center of the protein (14, 15). To determine whether catechol inhibited ribonucleotide reductase activity by chelating iron or by quenching the radical directly, we investigated the kinetics of iron chelation by catechol in Jurkat T cells by measuring the EPR signal at g = 4.3, which detects low m.w. iron chelates from various intracellular sources. The appearance of the catechol:iron complex was measured in the same samples used to measure tyrosyl radical content. Although a clear g = 4.3 signal was detected in cells treated with 40 μM catechol for 2 h, the catechol:iron chelate could not be detected in significant quantities following a 5-min exposure. These results suggest that while long term exposure to catechol may be associated with loss of iron from the M2 subunit, disappearance of the tyrosyl radical following short term exposure to catechol is due to quenching of the radical itself.

**Discussion**

These observations indicate that cigarette smoke extracts and their phenolic constituents, HQ and catechol, inhibit lymphocyte proliferation by quenching the essential tyrosyl radical in the M2 subunit of ribonucleotide reductase. Although the EPR experiments...
performed in these studies used a transformed human T cell line, we have previously shown that primary T cells are even more sensitive to the antiproliferative effects of HQ and catechol (9). The level of HQ and catechol in bronchoalveolar lavage fluid following cigarette smoke inhalation has not yet been determined. However, Hecht et al. (23) have shown that the typical smoker receives >100 μg HQ and catechol per cigarette. At a rate of 1.5 packs of cigarettes per day, this level of smoke would deliver sufficient HQ and catechol to produce 20 ml of 40 μM solutions every 30 min. Because the volume of fluid in the lungs is relatively small, it is likely that doses required for inhibition of M2 protein could be delivered by as few as two to three cigarettes, depending on tar content, and that lymphocytes may not have sufficient time to recover before a subsequent exposure.

Cigarette smoke contains HQ and catechol in roughly equal concentrations, and both clearly contribute to the antiproliferative effects of high-tar cigarette tar extracts. The high-tar extracts used in these studies contained 12 and 14 μM HQ and catechol, respectively, either of which can result in inhibition of 60% of the tyrosyl radical. Together, these levels of HQ and catechol alone can account for quenching of the radical following exposure to high-tar extracts, especially because the inhibitory effects on the M2 subunit were abolished when high-tar extracts were diluted 10-fold (data not shown). Notable however, is the level of quenching of the radical and inhibition of DNA synthesis by exposure to low-tar extracts containing only minimal amounts of HQ and catechol. Although it is likely that cigarette extracts contain additional redox active compounds that can inactivate the M2 subunit, none can be measured by reverse phase HPLC monitored by electrochemical detectors. One such agent may be NO, which is generated in cigarette smoke at levels up to 600 μg/cigarette, and has been shown to quench the tyrosyl radical in M2 protein (24).

The data presented here provide a molecular basis for inactivation of the M2 subunit by HQ and catechol. Other pharmacologic agents that interfere with nucleic acid biosynthesis, such as brequinar, mizoribine, and mycophenolate mofetil, have proved to be potent inhibitors of T cell responses. Although it is tempting to conclude that the antiproliferative effects of HQ and catechol are limited to inhibition of this enzyme, the potency of these phenolic compounds suggest that they modify additional pathways in lymphocytes. Indeed, HQ has been shown to inhibit activation of the transcription factor NF-κB and production of the T cell growth factor, IL-2 (25, 26). However, these effects cannot explain how low-tar cigarette smoke extracts, which contain minimal HQ or catechol and have little effect on the M2 subunit, also inhibit DNA synthesis. Preliminary studies in our laboratory indicate that cigarette smoke extracts block the G1 to S-phase transition, independent of their effects on ribonucleotide reductase. We are currently investigating the mechanisms of this cell cycle disruption following exposure to tobacco toxicants and its subsequent effects on lymphocyte proliferation.

Tobacco exposure has been implicated in disruption of the normal pathways of cell cycle control, which may affect both immune competence and tumor progression (27, 28). The potential effects of exposure to HQ or catechol cell cycle entry and progression have yet to be determined. However, HQ and catechol are known tumor promoters and have been shown to increase lung tumor invasiveness and metastasis in animal models (23, 29, 30). Recent reports suggest that both the M1 and M2 subunits of ribonucleotide reductase participate in cellular functions that are important for determining malignant potential (31, 32), and aberrant levels of ribonucleotide reductase expression and enzyme activity have been reported in human tumors (33–35). Additionally, HQ and catechol are highly redox active, which leads to the formation of reactive oxygen species, oxidative stress, and DNA damage (36–37). Thus, HQ and catechol may provide a selective advantage to malignant cells by promoting tumor cell growth and suppressing the immune response to those cells.

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