Induction of preneoplastic lung lesions in guinea pigs by cigarette smoke inhalation and their exacerbation by high dietary levels of vitamins C and E

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The development of effective chemopreventive agents against cigarette smoke-induced lung cancer could be greatly facilitated by the availability of suitable laboratory animal models. Here we report that male Hartley guinea pigs treated with cigarette smoke by inhalation twice a day for 28 days developed preneoplastic lung lesions, including bronchial hyperplasia, dysplasia and squamous metaplasia, analogous to those found in human smokers. The lesions were accompanied by increased expression of proliferating cell nuclear antigen and activation of the serine/threonine kinase Akt in the bronchial epithelium. In contrast, no lung lesions were found in guinea pigs (‘sham smoked’) that were submitted to identical procedures but without cigarettes. Compared with a diet low in vitamin C (50 p.p.m.) and vitamin E (15 p.p.m.), a diet high in vitamin C (4000 p.p.m.) and vitamin E (40 p.p.m.) significantly increased the incidence of these lesions. The inclusion of 1,4-phenylenebis(methylene)selenocyanate (p-XSC), a synthetic chemopreventive organoselenium compound, in the high vitamin C–high vitamin E diet at a level of 15 p.p.m. as selenium appeared to decrease the lesion incidence. Administration of (−)-epigallocatechin gallate, a powerful green tea polyphenolic antioxidant at 560 p.p.m. in the drinking water had no effect. As in human smokers, levels of ascorbate in blood plasma, lung, liver and the adrenal glands were significantly decreased by cigarette smoke inhalation. These results identify the guinea pig model of cigarette smoke-induced lung cancer, suggest that p-XSC may have activity as a chemopreventive agent against cigarette smoke-induced lung lesions and provide additional evidence that very high dietary levels of certain antioxidants can have co-carcinogenic activity in cigarette smoke-induced lung cancer.

Introduction

Lung cancer is the third most common cancer in humans and the leading cause of cancer deaths in the USA (1). The death toll is so high because lung cancer has a very high metastatic potential and thus has usually spread by the time it is detected (1). In addition, systemic therapies are generally ineffective against metastatic disease. More than 50% of new lung cancer cases develop in former smokers (2) and it is this group especially, as well as current smokers, which would benefit greatly from the availability of effective lung cancer chemopreventive compounds. Unfortunately, a serious obstacle to the development of chemopreventive agents against cigarette smoke-induced lung cancer is a scarcity of realistic animal models in which candidate chemopreventive, as well as therapeutic, agents could be evaluated.

We have been using the guinea pig to assess the effects of natural antioxidants, such as ascorbic acid (AA), alone and in combination with other antioxidants, on pulmonary damage caused by reactive oxygen and nitrogen species derived from inhaled cigarette smoke and from the consequent inflammatory events. The guinea pig provides a more suitable experimental model for this purpose than other species, such as the mouse, rat, hamster or ferret, which have also been used in the past for studies on effects of cigarette smoke inhalation (3–6), because the guinea pig, like the human, requires an exogenous source of AA (7). Thus, in the guinea pig it is possible to manipulate AA levels in organs through dietary means.

In the present work we describe the results of a 28 day cigarette smoke inhalation study in which preneoplastic lesions were induced in the lungs of guinea pigs fed diets high and low in AA and vitamin E (VE). These include hyperplasias, dysplasias and squamous cell metaplasias in the epithelia of bronchi and bronchiolae. Except for the ferret (4,8,9), this is the first time, to our knowledge, that early preneoplastic lesions which closely resemble those in human smokers (10) have been reported in a cigarette smoke-treated laboratory animal. Paradoxically, more lesions appeared in the lungs of guinea pigs consuming the high AA–high VE diet than the diet low in these antioxidant vitamins. Similar additive or synergistic effects of high levels of the antioxidant β-carotene and inhaled cigarette smoke on the induction of preneoplastic lung lesions were recently observed in the ferret (8,9). Because lesions appeared in the guinea pig lungs in the relatively short time of 28 days, the model we describe could be used to quickly assess the efficacy of putative chemopreventive agents or the relative harmful effects of different types of cigarettes.

We also describe the effects of two compounds with known chemopreventive activity on the formation of cigarette smoke-induced preneoplastic lesions. While the organoselenium compound 1,4-phenylenebis(methylene)selenocyanate (p-XSC) inhibited the decreases in body organ and blood plasma AA caused by cigarette smoke and appeared to have some effect in preventing smoke-induced lung lesions, the tea antioxidant (−)-epigallocatechin gallate (EGCG) appeared to be ineffective. These initial results indicate that the guinea pig model of

Abbreviations: AA, ascorbic acid; dehydro-AA, dehydroascorbic acid; EGCG, (−)-epigallocatechin gallate; HPLC-EC, high performance liquid chromatography with electrochemical detection; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; PCNA, proliferating cell nuclear antigen; p-XSC, 1,4-phenylenebis (methylene) selenocyanate; VE, vitamin E.
the human cigarette smoker we present here is viable for use in future chemoprevention studies.

Materials and methods

Animals

Male Hartley guinea pigs, 3 weeks of age, were purchased from Kingstar Farms (Kingston, NH). The animals were randomly divided into 12 groups of either 6 groups (1–6) or 5 groups (7–12). This uneven division into 5 or 6 animals per group was necessitated by the number of restrainers (11) on the Hamburg II inhalation device. While the total number of animals per group that could be incorporated into this study was also limited by the number of restrainers on the device, statistically significant results were nevertheless obtained (vide infra). The guinea pigs were housed in a laminar flow facility with a constant temperature of 21°C and 50% humidity, with alternating 12 h cycles of light and dark. The cages were changed every day. Groups 1–6 received a diet low in both AA (50 p.p.m.) and VE (15 p.p.m.). These groups are designated LC-LE. Groups 7–12 received a diet high in both AA (4000 p.p.m.) and VE (40 p.p.m.). These groups are designated HC-HE. Groups 1 and 2 and 7 and 8 were subjected to no further modifications of the diet (‘controls’). Groups 3 and 4 and 9 and 10 received the tea antioxidant EGCG at a level of 560 p.p.m. in the drinking water, which was supplied ad libitum. Groups 5 and 6 and 11 and 12 received p-XSC in their diet at a level of 15 p.p.m. as selenium. All animals were placed on their respective diets 2 weeks before the beginning of the study, for acclimatization, and were maintained on the diets for the duration of the study. The commercially prepared diets, which contained corn oil that had been stripped of tocopherols and a vitamin mixture without VE, were obtained from Dyets Inc. (Bethlehem, PA). The diets were subdivided into several portions and stored in plastic bags under nitrogen at –20°C to minimize the oxidation of AA. Diets were verified for AA and VE content by HPLC.

Chemicals

EGCG, ~94% pure by reverse phase HPLC with UV detection, was a generous gift of Dr John H. Weisburger, p-XSC, 99.9% pure, was synthesized by a method previously described (12).

Treatments

Groups 1, 3, 5, 7, 9 and 11 were exposed to cigarette smoke in Hamburg II respiratory devices twice a day (30 standard cigarettes in the device per smoking session) for 4 weeks. Groups 2, 4, 6, 8, 10 and 12 were enclosed twice each day for 4 weeks in the restrainers of a clean device, but were not exposed to cigarette smoke (‘sham controls’). The cigarettes used were University of Kentucky non-filter Reference Cigarettes 2R1.

All animals were weighed weekly and examined each day for signs of illness. All of the guinea pigs survived the 4 week study, however, incipient signs of scurvy and lower weight gains were noted in the low AA groups. The animals were killed by CO2 asphyxiation on the afternoon of the 28th day of the study. The guinea pigs were killed after CO2 anesthesia and lungs were perfused with 10% neutral buffered formalin at 1:100 in the same formulation of phosphate-buffered saline. The slides were incubated for 1 h at room temperature in a humid chamber. A horseradish peroxidase detection kit with 3,3′-diaminobenzidine was used for visualization and detection was accomplished with a Ventana ES automated staining system (Ventana Medical Systems, Tucson, AZ). A hematoxylin counterstain was used for contrast purposes. The slides were then dehydrated in graded alcohols and cleared in xylene. They were then mounted with synthetic resin and coverslipped. The PCNA labeling index was determined as the number of immunopositive cells multiplied by 100 and divided by the total number of cells per high powered field in tissue.

Immunohistochemistry of phosphorylated Akt

The immunohistochemical methods for detecting Akt phosphorylated at S473 have been described previously (13). After deparaffinization and hydration, antigen retrieval was performed in 10 mM Tris–HCl buffer, pH 8.0, for 10 min in a microwave oven (GE, 1200 W with turntable). Tissue sections were then incubated with anti-phospho-Akt (S473) antibodies (Cell Signaling, Beverly, MA) at 1:50 dilution overnight at 4°C. The binding of antibodies to their antigenic sites in the tissue sections was amplified with the use of biotinylated goat anti-rabbit antibodies and avidin–peroxidase conjugate for 30 min (Vectastain Elite ABC kits; Vector Laboratories Inc., Burlingame, CA), followed by reaction with 3,3′-diaminobenzidine (Sigma-Aldrich). Tissue sections were counterstained with Mayer’s hematoxylin (BioGenex Laboratories, San Ramon, CA).

Analysis of AA

AA in blood plasma was determined using paired ion, reversed phase high performance liquid chromatography with electrochemical detection (HPLC-EC), as described by Kutnik et al. (14). Isosorbide was used as an internal standard. To determine the levels of dehydroascorbic acid (dehydro-AA) in blood plasma, HPLC-EC was performed on plasma samples before and after the addition of diastrethoil to reduce dehydro-AA to AA (15). The difference between the two values obtained represents dehydro-AA. A Waters Model 990 photodiode array detector and an LC-4C amperometric controller (Bioanalytical Systems) were used and chromatographic data were processed using Empower Software from Waters. For the analysis of AA in tissues, the colorimetric assay as described by Zannoni et al. (16) was used. In this assay the tissue extract is mixed with H2PO4, α,α′-dipyridyl and FeCl3. The color produced by the coupling of α,α′-dipyridyl with Fe3+, generated by the reduction of Fe2+ by AA, is measured at 525 nm.

Statistical analysis

The histology and the PCNA data were compared among the groups using ANOVA followed by Tukey’s multiple comparisons procedure (17) to identify significant pairwise group differences. AA levels were compared among the groups using one-way ANOVA followed by Dunnett’s multiple comparisons (18) procedure where each treatment (EGCG and p-XSC) was compared with the control, separately for smoke-treated and sham groups. Student’s t-test was used to compare AA levels between smoke-treated versus sham groups, separately within each treatment group (control, EGCG and p-XSC).

Results

AA levels in blood plasma and selected tissues

Cigarette smokers are known to have ~15% lower blood plasma AA levels than non-smokers (19-21). Figure 1 shows that this phenomenon also occurs in guinea pigs, but is greatly intensified. In the case of guinea pigs fed the HC-HE diet (groups 7 and 8) plasma concentrations were decreased from 34.2 ± 6.9 to 4.7 ± 1.8 μg/ml by smoke inhalation, a difference of 86% (P < 0.0001). A similar, but somewhat smaller, decrease due to smoke inhalation occurred in guinea pigs fed the HC-HE diet with EGCG in the drinking water (groups 9 and 10), in which plasma concentrations decreased from 33.6 ± 7.7 to 5.9 ± 2.5 μg/ml, a difference of 82% (P < 0.0001). Of special interest, however, was the observation that the admixture of p-XSC into the HC-HE diet (groups 11 and 12) inhibited this effect: in these guinea pigs smoke inhalation produced a decrease from 32.7 ± 6.9 μg/ml in the sham smoked group (group 12) to 23.1 ± 3.6 μg/ml in the cigarette smoke inhalation group, only a 29% decrease (P < 0.05). Blood plasma levels of dehydro-AA in general paralleled
those of AA. Information on AA concentrations in plasma from guinea pigs consuming the low AA diets could not be obtained because the levels were below the detection limits of the HPLC-EC method used.

A similar lowering of AA by cigarette smoke inhalation was observed in the lungs, liver and adrenal glands (Figure 2). The colorimetric method of AA determination was used in these cases; the HPLC-EC method could not be used for tissue extracts because of the presence of large amounts of electrochemically active interfering material. In the lungs (Figure 2A) of the HC-HE-cont guinea pigs cigarette smoke produced a decrease in AA from 194 to 93.9 μg/g tissue, a 52% decrease (P < 0.05). In the animals consuming the HC-HE diet and EGCG in the drinking water a decrease in AA from 161 to 90 μg/g tissue (44%) occurred, however, this decrease was not statistically significant because of large inter-tissue variability. As in the plasma, the least effect of cigarette smoke inhalation on AA lung level was observed in the HC-HE-p-XSC guinea pig groups (groups 11 and 12), in which the decrease in lung AA was ~12%, from 277 in the sham group to 244 μg/g tissue in the smoke group; this was not statistically significant. Moreover, compared with the smoke-exposed HC-HE-cont and HC-HE-EGCG groups, the AA lung level (244 μg/g tissue) of the HC-HE-p-XSC smoked group was ~2.7 times higher (P < 0.0001). In guinea pigs consuming the LC-LE diet, only small differences in the AA content of lung tissues were observed; the levels of AA ranged from a low of 1.86 μg/ml in the sham-smoked LC-LE-EGCG group to a high of 8.1 μg/ml in the smoke-exposed LC-LE-p-XSC group.

Similar patterns of AA depletion by cigarette smoke inhalation and inhibition of this effect by p-XSC were observed in guinea pig livers and adrenal glands. In the liver (Figure 2B) the levels of AA in the HC-HE-cont and HC-HE-EGCG smoked groups were 63 and 60% less (P < 0.01), respectively, than in the corresponding sham smoked animals. In contrast, in the HC-HE guinea pigs consuming a diet containing p-XSC smoke inhalation induced only a 16% decrease, which was not statistically significant. In the adrenal glands (Figure 2C), which normally contain one of the highest concentrations of AA in the body, the results were analogous. In these organs the depletion of AA in the smoke-treated HC-HE-cont and

HC-HE-EGCG groups amounted to 61 (P < 0.0001) and 56% (P < 0.01), respectively. The sham smoked animals in these groups did not significantly differ in their AA levels from either the smoked or sham smoked guinea pigs receiving p-XSC in the diet (range of AA levels 1213–1504 μg/g). Again, there was essentially no difference in AA levels between the smoked and sham smoked guinea pigs receiving p-XSC. In the LC-LE groups the levels of AA ranged from 16 to 31 μg/g, with no statistically significant differences.
Pathological findings in bronchi and bronchiolar epithelium

Table I summarizes the histopathological findings in the lungs of the guinea pigs in the 12 different groups. Preneoplastic lesions comprised of epithelial hyperplasia, dysplasia and squamous metaplasia were found to various extents in every cigarette smoke-exposed group. In a given smoke-exposed group some animals had none, some had one and some had two or three lesions per 0.9 cm² lung section examined. To present the data in Table I the number of lesions per group was totaled and divided by the number of animals per group to obtain the mean and standard deviation. In sharp contrast to the smoke-exposed groups, not a single lesion was detected in any of the animals in the sham smoked groups. Thus, it is highly unlikely that the lesions were spontaneous, but were rather due to the cigarette smoke treatment. Of note, also, is the striking difference in the incidences of these lesions between guinea pigs consuming the LC-LE diets and those consuming the HC-HE diets. While a total of 12 lesions were observed in the HC-HE-cont smoke group, only 2 were found in the LC-LE-cont group, a difference significant at P = 0.01. While totals of 17 and 9 lesions were found in the HC-HE-EGCG and HC-HE-p-XSC groups, respectively, 2 and 1 lesions were observed in the corresponding LC-LE groups (P = 0.0001 and P = 0.05, respectively). Thus, we conclude that the HC-HE diet significantly stimulates the induction of preneoplastic lesions by cigarette smoke inhalation in guinea pigs. Although fewer lesions (n = 9) were found in the HC-HE-p-XSC group than in the HC-HE-Cont group (n = 12), suggesting that p-XSC may also inhibit the induction of preneoplastic lesions by cigarette smoke inhalation, the difference was not statistically significant. Examples of characteristic putative preneoplastic lesions from the lungs of cigarette smoke-treated guinea pigs are shown in Figure 3.

PCNA correlation with pathological findings

PCNA is the processivity factor of polymerase δ and an essential protein involved in DNA replication and DNA excision repair (22). It is widely used as a marker of dysregulated cell proliferation, providing results comparable to those obtained with [3H]thymidine or BrdU incorporation without the necessity of pretreating the animal (23). In humans increased PCNA expression has been used as a marker of bronchial metaplasia in smokers (24). Table II summarizes the percentage PCNA labeling indices obtained from paraffin-embedded tissue sections of guinea pig lungs. No statistically significant differences were detectable in PCNA labeling between the smoke-inhaling and sham smoked groups receiving LC-LE diets (groups 1-6). However, increases of 17.4 (P = 0.0001), 16.5 (P = 0.0001) and 12.7 (P = 0.01) in the labeling indices were observed in the HC-HE smoked groups 7, 9 and 11, respectively, compared with the HC-HE sham smoked groups (8, 10 and 12, respectively). There were no statistically significant differences among the three HC-HE smoked groups, but all three of these groups were significantly different from the LC-LE groups, with P values ranging from 0.001 to 0.0001. While the percentage labeling index of lung tissue from the HC-HE-p-XSC group was less than that of the HC-HE-EGCG or HC-HE-Cont groups, the differences were not statistically significant.

Correlation of Akt activation with cigarette smoke-induced lung lesions

Activation of the serine/threonine kinase Akt is a key step in the regulation of cellular processes that control tumorigenesis, including glucose metabolism, cell cycle progression and apoptosis (25). Akt is important in the biology of lung cancer. Akt is constitutively activated in non-small cell lung cancer cell lines (26), in which it antagonizes apoptosis, and active Akt has been detected in human lung cancer specimens. A role for Akt activation in lung carcinogenesis has been postulated because Akt is activated by tobacco components such as nicotine or the tobacco-specific carcinogen 4-(methylnitro-samo)-1-(3-pyridye)-1-butane (NNK) in normal human airway epithelial cells in vitro and increased Akt activation has been associated with phenotypic progression of NNK-induced lung lesions in A/J mice (13,27). Increased Akt activation has also been observed in dysplastic lung epithelial lesions from smokers (28). To determine if Akt was activated in the course
of development of lung lesions, we used phospho-specific antibodies against Akt to detect active Akt in an immunohistochemical analysis. As shown in Figure 4, higher levels of phosphorylated or active Akt were observed in the lesions that developed in the HC-HE smoked groups 7, 9 and 11, in comparison with the LC-LE smoked groups 1, 3 and 5 or the HC-HE sham smoked groups 8, 10 and 12. Although the incidence of lung lesions in the HC-HE p-XSC group was decreased, those lesions that did develop exhibited high levels of Akt phosphorylation (Figure 4I). Levels of phosphorylated Akt were not appreciably different between the LC-LE smoked and sham groups. These results show that the development of epithelial lesions in the HC-HE smoked groups is associated with two biochemical markers of proliferation and survival, namely increased expression of PCNA and activation of Akt.

Discussion

With the exception of the ferret (4,8,9), this is the first time that preneoplastic lung lesions similar to those appearing in the human smoker (10) have been induced by the inhalation of cigarette smoke in a laboratory animal. It is evident that attempts to induce lung cancer in rodents have not been successful (reviewed in 3,29). The fact that we observed preneoplastic lesions in all guinea pig groups (Table I) subjected to cigarette smoke inhalation for only 28 days suggests that, in contrast to other common laboratory animals, the guinea pig is more sensitive to cigarette smoke-induced lung neoplasms and that, with further development, it should furnish an excellent model for the human smoker and, importantly, the ex-smoker.

The induced guinea pig lung lesions bear a resemblance to smoking-induced human lesions not only in morphology but also in PCNA expression and the activation of Akt. Table II. PCNA labeling indices of guinea pig intrapulmonary bronchi and bronchiolar epithelium

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Exposure</th>
<th>n</th>
<th>PCNA labeling index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LC-LE-Cont</td>
<td>Smoke</td>
<td>4</td>
<td>35.5 ± 1.02</td>
</tr>
<tr>
<td>2</td>
<td>LC-LE-Cont</td>
<td>Sham</td>
<td>3</td>
<td>35.5 ± 0.24</td>
</tr>
<tr>
<td>3</td>
<td>LC-LE-EGCG</td>
<td>Smoke</td>
<td>3</td>
<td>36.4 ± 2.44</td>
</tr>
<tr>
<td>4</td>
<td>LC-LE-EGCG</td>
<td>Sham</td>
<td>2</td>
<td>32.1 ± 0.95</td>
</tr>
<tr>
<td>5</td>
<td>LC-LE-p-XSC</td>
<td>Smoke</td>
<td>3</td>
<td>30.6 ± 2.90</td>
</tr>
<tr>
<td>6</td>
<td>LC-LE-p-XSC</td>
<td>Sham</td>
<td>2</td>
<td>31.7 ± 5.76</td>
</tr>
<tr>
<td>7</td>
<td>HC-HE-Cont</td>
<td>Smoke</td>
<td>5</td>
<td>49.9 ± 2.67</td>
</tr>
<tr>
<td>8</td>
<td>HC-HE-Cont</td>
<td>Sham</td>
<td>4</td>
<td>32.6 ± 5.73</td>
</tr>
<tr>
<td>9</td>
<td>HC-HE-EGCG</td>
<td>Smoke</td>
<td>4</td>
<td>52.3 ± 4.35</td>
</tr>
<tr>
<td>10</td>
<td>HC-HE-EGCG</td>
<td>Sham</td>
<td>3</td>
<td>35.8 ± 1.31</td>
</tr>
<tr>
<td>11</td>
<td>HC-HE-p-XSC</td>
<td>Smoke</td>
<td>3</td>
<td>44.9 ± 2.25</td>
</tr>
<tr>
<td>12</td>
<td>HC-HE-p-XSC</td>
<td>Sham</td>
<td>3</td>
<td>32.2 ± 3.52</td>
</tr>
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</table>

aNumber of randomly chosen animals evaluated per group.
bSignificantly higher (P < 0.0001) than the corresponding sham group (Group 8).
cSignificantly higher (P < 0.0001) than the corresponding sham group (Group 10).
dSignificantly higher (P < 0.01) than the corresponding sham group (Group 12).

Preneoplastic lesions in guinea pig lung

![Fig. 3. Histopathology (hematoxylin and eosin) of bronchiolar epithelia of guinea pigs exposed to cigarette smoke. (A) Hyperplasia showing increased number of epithelial cells, stratification and basal cell hyperplasia (magnification ×20). (B) Severe hyperplasia exhibiting cellular pleomorphism with multiple layers and nuclear variation. Polarity of cells was lost (magnification ×20). (C) Dysplasia showing disorganization with hyperchromatic nuclei and an increased nuclear/cytoplasmic ratio. These changes involve almost the entire width. Variations of nuclear mitosis are found (magnification ×40). (D) Well-developed squamous cell metaplasia showing that the epithelium is transformed to stratified squamous cell epithelium (magnification ×40).](image-url)
from the HC-HE smoked group suggests that the vitamins may have played a role in activating Akt. Although a mechanistic connection between AA and Akt has not been described, VE can induce Akt phosphorylation and survival of neuronal cells (30). There are practical implications of the presence of active Akt in these preneoplastic lesions. Lung epithelial cells at intermediate steps in the transformation process are dependent upon the PI3K/Akt pathway for survival. Thus, agents that target Akt or components in the pathway may have utility in preventing the formation or progression of these smoking-induced lesions.

Several questions must be addressed in future studies. First, whether high VE is required for the stimulating effect of HC-HE on the formation of the preneoplastic lesions or whether the effect is due solely to AA. Since VE is believed to be involved in the recycling of AA (reviewed in 31), it may have an additive or a synergistic role with AA. Second, a dose–response effect of AA with AA-VE or VE must be established. Third, long-term studies must be carried out to test our expectation that the preneoplastic lesions described here will in fact progress to neoplasms. Carcinogenicity assays of tobacco-specific carcinogens (32) in this model should also yield basic but necessary mechanistic information and should answer the questions whether and why the guinea pig is apparently highly sensitive to the induction of lung tumors.

Recently the ferret was introduced as a laboratory animal that had potentially very good application in pulmonary toxicology in view of airway and anatomical similarities to the human (4,8,9,33). It is of no little interest that the consumption of high dietary levels of β-carotene by ferrets induces keratinizing squamous metaplasia in the lungs and that the incidence of these lesions is significantly increased by cigarette smoke exposure (9). These observations in the ferret are analogous to the results of two large epidemiological intervention studies (34,35), in which large doses of β-carotene, an antioxidant and a precursor of vitamin A, given to former or current smokers resulted in significant increases in the incidence of lung cancer. The increase in the induction of squamous metaplasia observed by Liu et al. (9) as a result of concomitant treatment of ferrets with high (but not low, ‘physiological’) levels of β-carotene and cigarette smoke is similar to our results with guinea pigs given high amounts of AA and VE. However, one major difference is that the administration of high amounts of β-carotene alone induced pulmonary lesions in the ferret (9), whereas in our study no lesions were observed in guinea pigs given high dietary levels of AA and VE without smoke exposure. Also, it appears that a longer time may be necessary for the induction of lung lesions in ferrets than in guinea pigs.

As another proposed animal model, Wang et al. (36) described the histopathology of squamous cell carcinomas of the lung induced by repeated skin painting of five different strains of mice with N-nitroso-tris-chloroethylurea, corroborating the earlier finding of Rehm et al. (37) and providing a highly interesting experimental system for the study of genetic modifiers of one of the major lung cancer types in human.

The decrease in AA levels in blood plasma and in the lung, liver and adrenal glands (Figures 1 and 2) of guinea pigs exposed to cigarette smoke is similar to the effect occurring

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<th>LC-LE</th>
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<th>EGCG</th>
<th>p-XSC</th>
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<tr>
<td>Smoked</td>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>Sham</td>
<td>D</td>
<td>E</td>
<td>F</td>
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<th>HC-HE</th>
<th>Cont.</th>
<th>EGCG</th>
<th>p-XSC</th>
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<tr>
<td>Smoked</td>
<td>G</td>
<td>H</td>
<td>I</td>
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<tr>
<td>Sham</td>
<td>J</td>
<td>K</td>
<td>L</td>
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Fig. 4. Immunohistochemical analysis of phosphorylated Akt expression in guinea pig lungs. Representative samples of lung epithelial tissues from each experimental group stained with phospho-S473 antibodies are shown. For discussion, see text.

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in the human smoker (19). This depletion of AA has been ascribed (38) to the reactions of AA with nitrogen oxides derived from NO, which is present in the gas phase of the smoke at high concentrations. The attenuation of this effect in guinea pigs consuming p-XSC may signify that p-XSC reacts faster with these nitrogen species than does AA. This does not seem to be the case with EGCG. Interestingly, a similar depletion of β-carotene was observed in ferrets exposed to cigarette smoke and fed a diet high in the antioxidant (39), and 4-nitro-β-carotene was identified as a major product of the reaction of β-carotene with nitrogen oxides in in vitro systems (40). We note that 4-nitro-β-carotene partially possesses the structure of a secondary nitroalkane. We have previously identified secondary nitroalkanes, such as 2-nitropropane, 2-nitrobutane and 3-nitropentane, as powerful carcinogens (41).

Because of its dependence on dietary AA (7), the guinea pig, like the human, but unlike other laboratory animals including mice, rats, hamsters and ferrets, is an appropriate species in which to examine the effects of AA deprivation and supplementation on biochemical factors related to disease. Many such studies have been reported, but few have focused on pulmonary damage and none, to our knowledge, examined the possibility that cigarette smoke might induce neoplastic or preneoplastic changes. In a work by Panda et al. (42) the authors determined oxidative damage to lung proteins and lipids in cigarette smoke-exposed, pair-fed guinea pigs receiving 0, 5 or 15 mg/day AA each. In this study the guinea pigs were exposed to the smoke for 7 days and oxidative damage was observed in the 0 and 5 mg AA groups, whereas complete protection was obtained in the group receiving 15 mg/day AA (equivalent to ~1500 p.p.m. in the diet). The protection against oxidative damage in the animals given the high AA dose was accompanied by a significant decrease in the AA levels of various tissues in cigarette smoke-exposed animals, an effect that we corroborate in Figure 2. The authors concluded that comparatively large doses of AA may protect human smokers from cigarette smoke-induced degenerative diseases, including cardiovascular disease and cancer (42). The differences between our present study and those of Panda et al. (42) include our use of a greater amount of AA, a higher dietary level of VE and a longer duration of smoke exposure.

The description of dietary AA as ‘high’ or ‘low’ requires some discussion. Examination of the literature makes it evident that many laboratories working with guinea pigs use their own judgment as to what represents ‘low’ or ‘high’ or ‘normal’ dietary AA levels. AA is essential in hydroxylase reactions for the formation of hydroxyproline and hydroxylysine in collagen (43). AA is also required (at least in vitro) in the hydroxylation of proline and asparaginyl residues of the hypoxia-inducible factor α subunit (44), which plays a major role in the vascularization of tumors and in cell proliferation. According to the Institute for Laboratory Animal Research (7), the normal requirement for AA by the guinea pig is 200 mg/kg diet, or 200 p.p.m. For a 300 g guinea pig consuming 20 g diet/day, this translates to ~13 mg/kg body wt. This dietary level of AA is adequate for normal collagen synthesis, wound healing and bone growth (7). Deficiency of AA shows up in reduced diet intake and weight loss followed by anemia and widespread hemorrhaging. According to the same source (7), the guinea pig requirement for VE is 26.7 mg/kg diet. A dietary deficiency in this vitamin can produce prostration, with severe body weight loss and the degeneration of skeletal muscle. In the present study we used a high level of 4000 p.p.m. AA in the diet, which is approximately equivalent to 267 mg/kg body wt/day AA. For a 68 kg human this would be ~18 g/day AA, clearly an unrealistic amount. We note, however, that unless otherwise specified by the purchaser, 4000 p.p.m. is the level of AA in the diet routinely supplied to laboratories using guinea pigs by Dyets Inc. To further complicate matters, it is known that both the human and the guinea pig show saturation kinetics for the absorption of AA from the intestine (45), and this saturation effect is also reflected in tissue AA levels. These considerations make it essential to perform AA dose–response studies in the future and to clarify the question whether high VE is necessary for the increase in bronchial preneoplastic lesions induced by cigarette smoke. With regard to the dose of cigarette smoke, from analyses of cotinine in the guinea pig urine on day 28 of the study (results not shown) the amount of smoke inhaled was comparable to that of a human smoking one pack of cigarettes per day, assuming that the metabolism of nicotine in guinea pig and man is similar.

The mechanism by which a diet ‘high’ in AA and VE enhances the production of preneoplastic lesions in the lungs of guinea pigs treated with cigarette smoke by inhalation is, at present, a matter of speculation. Several possibilities may be suggested. For instance, high AA and/or VE intake might increase the levels of enzymes catalyzing the metabolic activation of tobacco-specific carcinogens such as NNK and benzo[a]pyrene. A different mechanism is suggested by the observation that oxidation products of AA, such as l-threose, undergo so called ‘glycation’ reactions with amino groups of proteins both in vitro and in vivo (46). Such adduct formation could result in changes in the biological function of transcription factors and other proteins and enzymes involved in apoptosis and cell cycle control, resulting in dysregulation and tumor promotion or co-carcinogenicity. The in vitro formation of Lys–Arg, Lys–His and Lys–Lys cross-links in proteins incubated with ascorbic acid has also been described (47). The modification of histones in this way could decrease the expression of tumor suppressor genes. We are presently looking into some of these and other possibilities.

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References