Review

Epidemiological Models of Carcinogenesis: The Example of Bladder Cancer

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Abstract

Epidemiological studies have clearly shown that smokers have an increased risk of bladder cancer. Chemical, biochemical, and molecular investigations indicate that such risk might be due to aromatic amines which are present in tobacco smoke. In particular, collaborative studies have shown that smokers have increased levels of hemoglobin-4-aminobiphenyl adducts in their blood and that these levels show a dose-response relationship and an association with the most carcinogenic variety of tobacco, air-cured or black tobacco. Adduct concentrations were also modulated by the genetically based slow acetylator phenotype. In addition, investigations in dogs and humans have described a DNA adduct in bladder biopsies and in exfoliated bladder cells that is a derivative of 4-aminobiphenyl. This paper summarizes the epidemiological, biochemical, and molecular evidence concerning the possible mechanisms of bladder cancer induction in smokers and in occupationally exposed workers. The case of bladder cancer is an example of integration between epidemiological studies, mathematical modeling, and laboratory investigations aiming at the elucidation of mechanisms of carcinogenesis.

Introduction

Through experimental examples, Cohen and Ellwein (1) have suggested that cell proliferation may be a crucial step in bladder carcinogenesis, in addition to events that induce mutation. This suggestion is not new, being included also in the epidemiological model of carcinogenesis proposed by Moolgavkar (2). The latter model is based on two mutational events, with an intermediate step of increased cell proliferation. Exposures can act by increasing the rate of the first (initiation) or second (progression) mutation, or through the balance between cell death and cell division. The model has been successful in at least three, qualitatively different, instances.

Human Retinoblastoma. The need for two mutations has been clearly shown, although there is no evidence of any chemical exposure which might be a risk factor for this cancer (3).

Lung Cancer and Cigarette Smoking. The most recent analyses of epidemiological data sets clearly suggest that the effect of timing of smoking habits (age at start, age at cessation) fits better with Moolgavkar’s model than with others. In this case the relevant chemical exposure has been identified, although it is a complex mixture rather than a single chemical.

Animal Experiments Based on Skin Painting. Several experiments have shown that two mutations are necessary, one at the “initiation” stage, leading to benign papillomas, and one at the “conversion” stage, leading to carcinomas. The role of promoters is proposed to be exerted in between the two mutations, and mainly consists of a proliferative stimulus.

The problem with mathematical models is that most of the available data sets are usually compatible with different models, and the choice between two or more of these is based on statistical aspects (i.e., “goodness of fit”), which have nothing to do with the available biological knowledge.

To make more credible inferences to be used in “risk assessment” procedures, the integration of both epidemiological and biological data is absolutely needed. In particular, the following types of information should become available, if possible, for at least one risk factor and the corresponding cancer site: (a) shape of the dose-response relationship; (b) effects of timing of exposure (age at start, age at cessation, pattern of risk after exposure); (c) internal dosimetry (adducts); (d) experimental data on the putative mechanism of action (genotoxic/epigenetic); (e) other relevant data on important effect modifiers, like genetically based metabolic polymorphism.

The relationship between lung cancer and cigarette smoking is probably the best-known demonstration of epidemiological patterns such as the dose-response relationship and effect of timing of exposure. However, there is no evidence available suggesting that one particular component of tobacco is responsible for the lung carcinogenicity of smoking. The state of knowledge thus far has been too incomplete to use an individual chemical (or a chemical class) as a “marker” to study the biological mechanisms of lung carcinogenesis. However, there is increasing evidence of a possible mechanism of induction of bladder cancer by arylamines, which is at the moment the most comprehensive example of this type.
Role of Arylamines in Bladder Carcinogenesis: Epidemiological Evidence

It is well known that occupational exposure in dye-producing industries to high levels of arylamines, such as benzidine, 2-naphthylamine, and 4-aminobiphenyl, was followed by enormously increased risks of bladder cancer, on the order of 50- to 60-fold or more (4). Tobacco smoking is also a risk factor, being responsible for about 50% of bladder cancers in males living in Western countries (5). It can be reasonably hypothesized that what makes tobacco smoke carcinogenic to the bladder is its arylamine content. Table 1 shows the concentration of several arylamines in smoke from air-cured and flue-cured tobaccos (6). Doll et al. (7) estimated in 1972 that the risk of a light smoker of flue-cured tobacco was grossly comparable to the risk of a gasworker exposed to arylamines, at a concentration much lower than that found in dye-producing industries. In both cases (smokers and gasworkers) the estimated exposure to 2-naphthylamine was around 100 μg in the course of 20 years, and the relative risk was around 2.

Table 1 suggests that smoking air-cured instead of flue-cured tobacco entails exposure to a dose of arylamines which is around 2 to 2.5 times higher; therefore, one would predict that smokers of air-cured tobacco have a relative risk of about 4 to 5. The latter figure has been reported, in fact, in three studies in which detailed information on the type of tobacco was available (8–10). In other words, epidemiological studies in Latin countries clearly suggest that air-cured tobacco is more carcinogenic to the bladder than flue-cured tobacco, and this difference may be attributed to their different content of arylamines. Therefore, one can make the assumption that smoking is carcinogenic to the bladder because it contains arylamines. Although this hypothesis may be too rough, it can be a useful simplification. In the following I will use arylamines as a marker to study the mechanisms of bladder carcinogenesis. In particular, 4-aminobiphenyl will be considered, because (a) among the different arylamines it is one of the most potently carcinogenic; (b) among different arylamines, 4-ABP1 has the highest covalent binding index; i.e., it seems to have the strongest ability to form complexes (adducts) with macromolecules including hemoglobin; (c) 4-ABP is the best-studied arylamine from the perspective of "molecular epidemiology" (see below); and (d) 4-ABP is subject to a metabolic pathway (N-acetylation) which is well known to have a polymorphic distribution in the population (see below).

Evidence for Metabolic Polymorphism as an Effect Modifier

N-Acetyltransferase is an enzyme involved in the deactivation of some arylamines, the urinary excretion of which is increased after acetylation. Human populations show a characteristic genetically based polymorphism for the activity of this enzyme, with about 50% of subjects being "slow" acetylators and 50% "fast" acetylators. In their classic report, Cartwright et al. (15) described a hospital-based case-control study of 111 bladder cancer cases and 207 controls (one group of healthy subjects recruited in London and one group of urological patients in Huddersfield). The N-acetylation phenotype was assessed by the measurement of the monoacetylatedp:sonedapsone:da:soneratio, and 0.3 was used as the cut point between the phenotypes. The proportion of "slow acetylators" was 57% among the controls and 67% among the cases. However, this proportion was strikingly increased in a group of 23 occupationally exposed cases

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1 The abbreviation used is: 4-ABP, 4-aminobiphenyl.

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Role of Arylamines in Bladder Carcinogenesis: Evidence from "Molecular Epidemiology"

There are at least four sources of information that indicate 4-aminobiphenyl is relevant to bladder carcinogenesis in smokers: studies on hemoglobin adducts; studies on DNA adducts in exfoliated bladder cells; studies on bladder biopsies in humans; and studies on bladder biopsies in dogs. Talaska et al. (11) have shown that the administration of 4-ABP to dogs resulted in the formation of a main DNA adduct, N-(deoxyguanosin-8-yl)-4-aminobiphenyl, in bladder cells. Subsequently, they studied DNA adducts in the biopsies of 42 subjects with bladder cancer and again found that N-(deoxyguanosin-8-yl)-4-aminobiphenyl was one of the main adducts in smokers (12). Finally, one comprehensive investigation was conducted among 97 volunteers, measuring the levels of the 4-aminobiphenyl adducts with hemoglobin and with DNA in exfoliated bladder cells. The results show that hemoglobin adducts clearly correlate with the number of cigarettes smoked, with urinary cotinine and nicotine, and with the type of tobacco (air- or flue-cured) (13, 14). In addition, 2 of 12 DNA adducts found among smokers were clearly correlated with both the number of cigarettes smoked and the concentration of 4-ABP-hemoglobin adducts; one of the two was quite similar to N-(deoxyguanosin-8-yl)-4-aminobiphenyl. There are good reasons to believe, therefore, that 4-ABP, and perhaps other arylamines, may play an important role in smoking-induced bladder carcinogenesis.
(with probable exposure to benzidine). In fact, 22 of 23 (P = 0.0005) were slow acetylators; in addition, the only fast metabolizer had an adenocarcinoma, i.e., a histological type different from the other 22 (transitional cell carcinomas). The proportion of virtually 100% slow acetylators among occupationally exposed bladder cancer cases is the highest reported in the literature; all the procedures were blind, and the method used for phenotyping is simple and accurate.

Other hospital-based case-control investigations on bladder cancer have been reported, in which controls are usually patients with urological conditions or healthy volunteers. Methods used for phenotyping were based on sulfadimidine or sulfamethazine (for a review see Ref. 16). Table 2 reports the proportions of slow acetylators in cases and controls. Overall, these data are an argument for a small (30–50%) increase in the proportion of slow acetylators in bladder cancer patients, particularly among arylamine-exposed workers. However, the slight elevation of risk is also compatible with bias.

In addition to these data concerning bladder cancer, information from "molecular epidemiology" is also available. In the study on 97 volunteers mentioned above, the concentration of 4-ABP-hemoglobin adducts was clearly higher in slow acetylators, as Table 3 shows. When smoking habits (dose, type of tobacco) and the metabolic phenotype were included in a multivariate model, an independent and statistically significant contribution by the latter was found. In addition to this direct evidence of a role played by the phenotype for N-acetylation in the concentration of adducts, there is also indirect, but quite suggestive evidence of polymorphism. We have analyzed the hemoglobin adducts formed by 14 different arylamines which were the object of a first pilot study among smokers. After controlling strictly for smoking habits, we analyzed the residual interindividual variability for the adduct concentration, by the means of factor analysis; the aim was to identify potential sources of variability not explained by smoking. The result was surprising, since the adduct concentration varied considerably according to the type of arylamine (mono- or binuclear). In fact, adducts from binuclear arylamines (2-naphthylamine, 4-aminobiphenyl, 3-aminobiphenyl) were highly correlated reciprocally, and so were adducts from mononuclear arylamines (all the others). This is strongly suggestive of a metabolic polymorphism in the study population that is responsible for interindividual variability not explained by smoking habits (17).

### Dose-Response Relationship

The shape of dose-response relationships in cancer epidemiology has been interpreted in at least two different ways: (a) to infer the number of stages, within the frame of multistage carcinogenesis; (b) to make inferences about saturation or induction of enzymes involved in the metabolism of carcinogens. An example of the first inference is the observation of a quadratic dose-response relationship between the number of cigarettes smoked and the risk of lung cancer, and the ensuing inference that tobacco smoke acts on two stages of the carcinogenic process in the lung (18). An example of the second type is the observation of a convex dose-response relationship after experimental administration of some carcinogens to animals, which was interpreted as an expression of enzyme saturation. It is clear that both types of inference are rather arbitrary, if based on the simple observation of the shape of the relationship. At least a third possibility exists, i.e., that the dose-response relationship is an expression of polymorphism within the study population, indicating the presence of subgroups with different susceptibilities to the action of carcinogens.

Cohen and Ellwein (1) have inferred from the observation of bladder cancers experimentally induced with an arylamine (2-acetylamino-2-naphtylamine) that this chemical would act on two mutational stages and stimulate cell proliferation at high doses. In practice, the increase of tumor prevalence was very slow and about linear at low doses, while at high doses the dose-response curve became much steeper. The authors conclude that, in addition to the two mutations induced at all doses, 2-acetylamino-2-naphtylamine at high doses would also have an effect on urothelial hyperplasia.

As far as epidemiological studies of bladder cancer are concerned, rather than an exponential shape, a convex dose-response relationship between the amount of cigarettes smoked and the relative risk has been repeatedly observed. In other words, the relative risk increases quickly and then seems to reach a plateau. For example, in the largest case-control study the relative risks by

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number of cigarettes smoked (nonsmokers were the reference category) were: <20, 1.8 (based on 658 cases); 20-39, 2.6 (1102 cases); and 40+, 2.6 (392 cases) (19). In a Danish investigation, the following relative risks were found: 1-14, 4.2; 15-29, 4.9; and 25+, 4.3 (20). In other investigations, however, the demonstration of a plateau is less dramatic. No data are available for occupationally exposed cohorts. A convex (less than linear) dose-response relationship has also been observed when correlating the concentration of 4-ABP-hemoglobin adducts with markers of recent smoking (number of cigarettes, urinary cotinine and nicotine) (14, 21). This kind of relationship could be expected a priori if the population would include two subgroups, one metabolizing rapidly and one metabolizing slowly the relevant chemical. In fact, when the hemoglobin adduct data were subdivided according to the metabolic phenotype (slow or fast acetylator), the picture reported in Table 4 emerged. It is clear that slow acetylators have a higher level of adducts at low levels of smoking, and the concentration does not increase subsequently; on the contrary, the curve for fast acetylators is more regularly increasing. The association of the two curves leads to a less-than-linear relationship. Indeed, the regression coefficient of 4-ABP-hemoglobin adducts versus nicotine plus cotinine in the urine was 6.5 (SE, 3.5; P = 0.07; smokers only); however, when the square root of the adduct was used, the regression coefficient became 23.0 (SE, 11.1; P = 0.04). A square-root transformation, therefore, gave a better fit of the curve, which can be interpreted as a mixture between two subpopulations of curves. Unfortunately, this observation is based on small numbers, as expressed by the large SE in Table 4, and warrants further investigation.

It would be a simplification to conclude that the convex dose-response relationship, observed between the number of cigarettes smoked and the relative risk of bladder cancer, indicates the same phenomenon as hypothesized for hemoglobin adducts. In fact, (a) carcinogenesis is more complex than the simple binding of arylamines to macromolecules; and (b) the concentration of 4-ABP-hemoglobin adducts is measured on a linear scale, while the relative risk is on a multiplicative scale, being an expression of incidence ratios. Nevertheless, the similarity of the dose-response curves is suggestive. In any case, it is unlikely that the trend with dose observed in humans is compatible with the observations made in animals treated with 2-acetylaminofluorene; i.e., it is unlikely that tobacco smoke acts on two mutational stages and induces urothelial hyperplasia, as hypothesized by Cohen and Ellwein.

### Timing of Exposure and Risk of Bladder Cancer: Smoking and Occupational Exposure

In the case of cigarette smoking, increasing age at start seemed to be associated with decreasing relative risks in a few investigations (10, 19), with no information on the type of tobacco. In another study, this pattern was evident for smokers of air-cured tobacco but not for smokers of flue-cured tobacco, after allowing for time since quitting. In addition, switching from the former to the latter type did not change the risk substantially in comparison with smoking air-cured tobacco throughout life, indicating a more important effect of the air-cured variety early in life (Table 5) (8).

Quitting smoking is associated with a dramatic drop in the risks of bladder cancer (8, 10, 19), irrespective of the type of tobacco. This is a clear suggestion of a late-stage action. In the two studies which considered separately by tobacco type the effect of quitting, the relative risk remained well above the level of nonsmokers only for smokers of the air-cured variety, suggesting also an early-stage activity. In Table 5 the relative risks for smokers of air-cured tobacco who quit smoking are around 2-2.5, irrespective of their temporary switching, or not, to blond tobacco; no information was available for subjects starting with the blond variety and then switching to the black one.

The study of time-related variables is complex, since they are strictly correlated. Only if a large proportion of the subjects stopped smoking and then started again several times, a distinction between duration, age started, and time since quitting can be made on statistical grounds; usually, however, such a distinction cannot be made (22). The overall picture concerning the type of tobacco and bladder cancer, as far as time-related variables are concerned, seems to be the following. In general, tobacco smoking mainly exerts a "late stage" action, as is clearly indicated by the rapid drop in risks after discontinuation. Air-cured tobacco, however, seems to have an "early stage" action, as suggested by (a) the stronger association with age at start; (b) the fact that the relative risks remain high after 10 to 15 years since quitting; and (c) the fact that switching to flue-cured tobacco does not seem to be different from continuing to smoke air-cured tobacco.

| Table 4 | Levels of 4-aminobiphenyl-hemoglobin adducts according to levels of nicotine plus cotinine in the urine: smokers only |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Cotinine + nicotine (µmol/mmol) | 4AB adducts (pg/g) | Mean | SE |
| Slow acetylators only | | | |
| <0.5 | 153 | 41 |
| 0.5-1.4 | 114 | 17 |
| 1.5-2.4 | 134 | 15 |
| 2.5+ | 148 | 20 |
| Fast acetylators only | | | |
| <0.5 | 44 | 8 |
| 0.5-1.4 | 66 | 12 |
| 1.5-2.4 | 92 | 36 |
| 2.5+ | 121 | 16 |

* One subject only.

| Table 5 | Effect of switching from black to blond tobacco* |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Black tobacco throughout life | Black tobacco switching to blond |
| Current | Former | Former black, current blond | Formerly black, later blond, then quitting |
| OR** | 6.2 | 2.1 | 5.5 | 2.5 |
| 95% CI | 3.3-11.7 | 1.1-4.0 | 3.2-9.5 | 1.3-5.0 |

* Ref. 8.
** Adjusted for age and duration.

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OR: odds ratio; CI, confidence interval.
Table 6 compares the effect of age at start and years since cessation for both air-cured tobacco smoking and occupational exposure to highly carcinogenic arylamines. Incidentally, the two studies (one case-control and one cohort study) have been conducted in the same geographic area, the province of Toronto. The first data set, coming from a case-control study, also includes 10 bladder cancers which occurred among the dye workers followed in the cohort study (however, there was no confounding effect of occupational exposure on the smoking estimates or vice versa). Although the workers were exposed to much higher doses, as reflected by a relative risk of 101 among those currently exposed (based on 15 observed bladder cancers versus 1.1 expected), the trends of relative risk with increasing age at start and with years since cessation are strikingly similar for air-cured tobacco smokers and the workers occupationally exposed. The conclusions of the authors of the cohort study were that occupational exposure to arylamines acted on two stages, one early and one late (23).

Conclusions and Perspectives
I have made a series of inferences which need further support from the data: (a) that the bladder carcinogenicity of smoking is mainly due to arylamines, which undergo genetically based metabolic polymorphism; (b) that the role played by arylamines and the metabolic polymorphism for N-acetylation might explain the similar dose-response relationship which has been found for both the risk of bladder cancer and the concentration of 4-ABP-hemoglobin adducts in the blood of smokers; (c) that, again, the role played by arylamines in cigarette smoke might explain the similarities between smokers and occupationally exposed workers in the effects of exposure timing (age at start, age at cessation). A further inference might be that arylamines (which include potent mutagens, like 2-naphthylamine and 4-aminobiphenyl) act on two mutational stages in the carcinogenic process, i.e., inducing mutations of the ras or p53 genes. Although this is only a model, I believe it has the advantage of being based on both mathematical treatment of epidemiological data and the use of the biomarkers of internal dose and susceptibility. Any model is a temporary pictorial representation of reality, and its success is revealed by its ability to make predictions and by the confirmation of such predictions.

References