

Role of Genotoxic and Nongenotoxic Effects in Multistage Carcinogenicity of Aromatic Amines

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It has been demonstrated in several model systems that tumors arise in a multistage process. Carcinogenic aromatic amines are complete carcinogens, which usually produce tumors in typical target tissues without any additional treatment. The tissue specificity, however, cannot readily be explained by genotoxic effects, and the role of secondary effects is not well understood. Promotional pressure on initiated cells can be produced by endogenous factors but also by the chemical itself. Comparison of the effects on rat liver of 2-acetylaminofluorene (AAF) and *trans*-4-acetylaminostilbene (AAS) provides some evidence that initiating and promoting properties of these chemicals can be separated. AAS is a strong initiator in rat liver but seems to lack promoting activity; AAF is a less efficient initiator but has tumor promoting properties. The results obtained so far indicate that promoting pressure is not produced by the acute, cytotoxic effects of AAF. It is therefore concluded that nongenotoxic, possibly receptor-mediated effects are involved.

Introduction

Many aromatic amines are complete carcinogens and produce tumors in typical target tissues. Tissue specificity and the latency period cannot be correlated well with genotoxic effects as measured by DNA binding and the generation of particular adducts (1). For instance, *trans*-4-acetylaminostilbene (AAS) generates more DNA adducts in the liver than in any other tissue of rats but produces liver tumors only when additional promotional pressure is applied (2). By definition, therefore, this chemical is a tumor initiator in rat liver. 2-Acetylaminofluorene (AAF), on the other hand, is able to produce liver tumors without additional promotion, despite the fact that it binds less efficiently to DNA than does AAS and is less mutagenic in many *in vitro* test systems. It is therefore unlikely that a particularly strong initial genotoxic effect starts the whole process of tumor formation in this case. Repair is also not an adequate explanation for the differences because both chemicals produce persistent DNA adducts (3). We therefore assume that AAF, in contrast to AAS, has some additional properties that create promotional pressure. We have previously collected data that sup-

port this working hypothesis (4) by comparing the effects of these chemicals in the rat liver system at the biochemical and the biological level, and we now summarize some more recent results.

DNA Adducts *In Vivo*

DNA adducts of AAF are well characterized (5), and there is evidence that *N*-(deoxyguanosine-8-yl)-2-aminofluorene accumulates after repeated uptake and represents a promutagenic lesion. With AAS the situation is much more complicated. The reaction of *N*-acetoxy-AAS, a model of the ultimate reactive form, with guanine yields a whole series of adducts, some of which have been identified (6). Particularly interesting are two types of cyclic adducts that are formed by the addition of the stilbene double bond to either N1 and N2 or N2 and N3 of guanine. Preliminary findings indicating that the latter form (called B adducts in previous reports) is also formed *in vivo* have now been confirmed. The four isomers are clearly present in RNA and DNA. The major adduct in RNA has not yet been identified. This major adduct accounts for up to 30% of radioactive material and contains the acetyl group of AAS. This adduct is present in DNA only in small amounts. The most difficult problem in analyzing DNA adducts is the vast amount (up to 70%) of nonhydrolyzable material eluting early from Sephadex LH20 columns. Neither by additional enzymatic treatment nor by chemical hydrolysis

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under depurinating conditions was it possible to degrade this material further. Whether this is due to intrastrand or interstrand crosslinks (7) remains to be demonstrated. AAS is certainly strongly genotoxic, and it is assumed that its genotoxicity is a result of adduct formation.

AAS As an Initiator in Rats

AAS is a complete carcinogen for rats and produces tumors quite selectively in Zymbal glands (8). DNA binding has been demonstrated in many tissues (9), reaching the highest levels in liver and kidney. The DNA adducts formed in these tissues must be able to produce critical lesions and thus initiated cells because promotional pressure with appropriate secondary treatments results in tumor formation (Table 1).

AAF and AAS As Initiators in Rat Liver

According to the classical concept, a synergism of carcinogens should be restricted to chemicals with a common target tissue. Thus, 4-dimethylaminoazobenzene and diethylnitrosamine, two liver carcinogens, act synergistically (12), but *trans*-4-dimethylaminostilbene and diethylnitrosamine, in which one of the constituents is known to produce ear duct tumors and the other liver tumors (13), do not act synergistically. In light of the fact that AAS initiates rat liver cells, the synergism concept has to be reevaluated by concentrating on the more refined question of whether DNA lesions induced by different chemicals may be additive and result in a respective level of initiation. This was shown to be the case with AAS and AAF (14). In addition, AAF turned out to be a significantly weaker initiator than AAS; a finding that is supported by the experiments described below.

AAF As a Promoter

The most surprising result of the initiation-promotion experiments (Fig. 1A) was that the sequence of administration during initiation influenced the results. AAF and AAS were administered sequentially to adult rats within 4 weeks. This initiation phase was followed by partial hepatectomy and phenobarbital in the drinking water as a promoting regime. When AAS was admin-

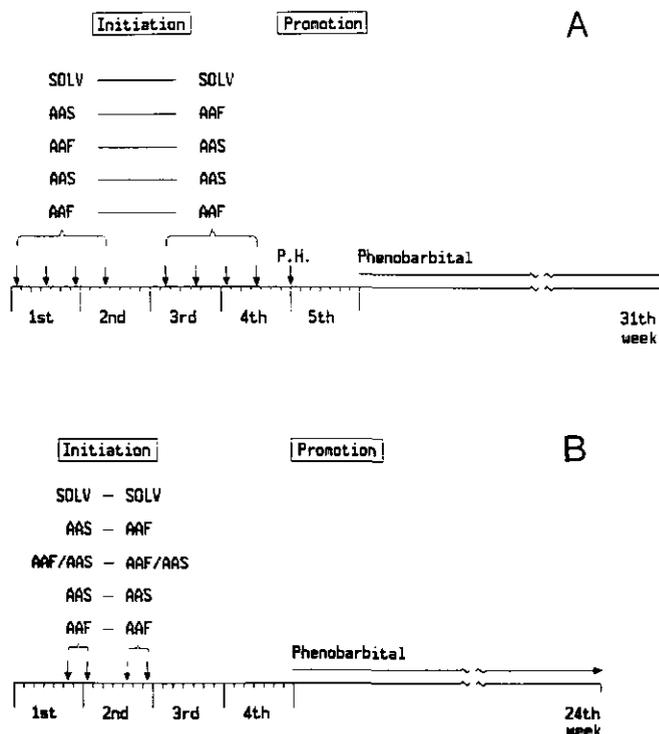


FIGURE 1. Protocol for initiation-promotion experiments in rat liver. (A) Administration of initiators 2×4 doses by gavage to adult animals. P.H., partial hepatectomy. Phenobarbital, 250 ppm in the drinking water. (B) Oral administration of initiators 2×2 doses in condensed milk to newborn animals. Phenobarbital, 500 ppm in the drinking water. The AAF/AAS group received the same total dose of AAS and AAF as in the AAS-AAF group, but as a mixture.

istered first and AAF second, preneoplastic lesions grew faster and became larger than when the compounds were applied in the reverse order, despite the identical promoting treatment. This can hardly be explained by the lower genotoxic effects of AAF and appears to be typical of the promoting effects of AAF on AAS-initiated cells.

Promoting effects of AAF were frequently used in the initiation-selection protocols of Farber and his associates (15). Promotion was attributed to growth inhibitory, cytotoxic effects of AAF on the surrounding normal cells, which are thought to be damaged to a greater degree than preneoplastic cells with their improved capacity to inactivate xenobiotics (16,17).

We began to study the cytotoxic properties of AAF in isolated perfused rat liver as well as in intact animals with bile fistulas, but so far we have not been able to find any adequate biochemical indicators for cytotoxicity. Some of the parameters measured are summarized in Table 2. The following quantitative considerations support the view that acute toxic effects are not likely to be involved in liver tumor production by AAF. The dose rate of AAF can be estimated to be about $2 \text{ nmole/min} \times \text{g liver}$ with 0.02% AAF in the diet, which corresponds to a dose of about $0.05 \text{ mmole/kg} \times \text{d}$. In

Table 1. Role of secondary treatment for the generation of tumors following initiation with AAS in female Wistar rats.

| Secondary treatment | Tumor | Reference |
|------------------------|---------------------------------------|-----------|
| None | Zymbal gland | |
| Zymbalectomy | Sebaceous glands in lips and eye lids | (10) |
| Partial hepatectomy | Liver | (2) |
| Phenobarbital | Liver | |
| Unilateral nephrectomy | Kidney | (11) |
| Cyclodextrin | Kidney | |

Table 2. Indicators for acute toxicity measured in livers of rats with bile fistula (*in vivo*) dosed with up to 1 mmole/kg AAF, or in isolated perfused livers with concentrations up to 50 μ M AAF.

| Parameter | Effect ^a | |
|--------------------------|---------------------|-------------------------|
| | <i>In vivo</i> | Isolated perfused liver |
| Enzyme leakage | | |
| GPT in serum | ± | |
| LDH in perfusate | | ± |
| Liver GSH | 55% | |
| Oxidative stress | | |
| GSSG in bile | ± | ± |
| Redox state | | |
| Lactate/pyruvate | | ± |
| Lipid peroxidation | | |
| Thiobarbiturate positive | ± | ± |

^a(±) Indicates no effect; not significantly elevated.

isolated perfused liver, the maximum concentration of AAF was limited to 50 μ M due to its low solubility. This amounts to a dose rate of 200 nmole/min \times g liver, which is about 100 times more than during tumor production. With intact animals, doses up to 1 nmole/kg were injected IP, corresponding to a dose rate of about 20 nmole/min \times g liver, which is still about 10 times as much as during tumor generation. We therefore conclude that AAF has promoting properties that are not related to the formation of reactive metabolites or reactive oxygen and may be nongenotoxic in nature (18).

Synergism of AAF and AAS

The question of synergism in a multistage process is difficult to answer. Early markers such as γ -glutamyl-transpeptidase (GGT)-positive foci in the initiation-promotion experiments mentioned above (14) after 14 and 27 weeks of promotion demonstrated effects that were clearly stronger than expected from adding the effects produced by the same dose of each compound administered alone during the same period as in the combination groups. This was interpreted as indicating a more than additive initiating effect of the combinations. However, one could argue that the lapse of time between the last administration of the initiator and the beginning of the promoting regime could also influence the outcome. In the new experiments, therefore, different control groups were used. Two series of the same initiator were given and compared with both sequences of the combination of one series of each initiator (Fig. 1A).

The effects were measured after 13 and 26 weeks of promotion using the expression of placental glutathione transferase (GST-P), GGT, and morphologically discernible foci in hematoxylin-eosin-stained sections as markers (Fig. 2). The number of foci per square centimeter, the average size of foci (mm^2), and the area of foci in o/oo of the total area screened were calculated. The different markers yielded slightly different results. With GST-P, smaller foci could be clearly identified, and thus the number of foci is greater than with the other markers. Morphological alterations represent more ad-

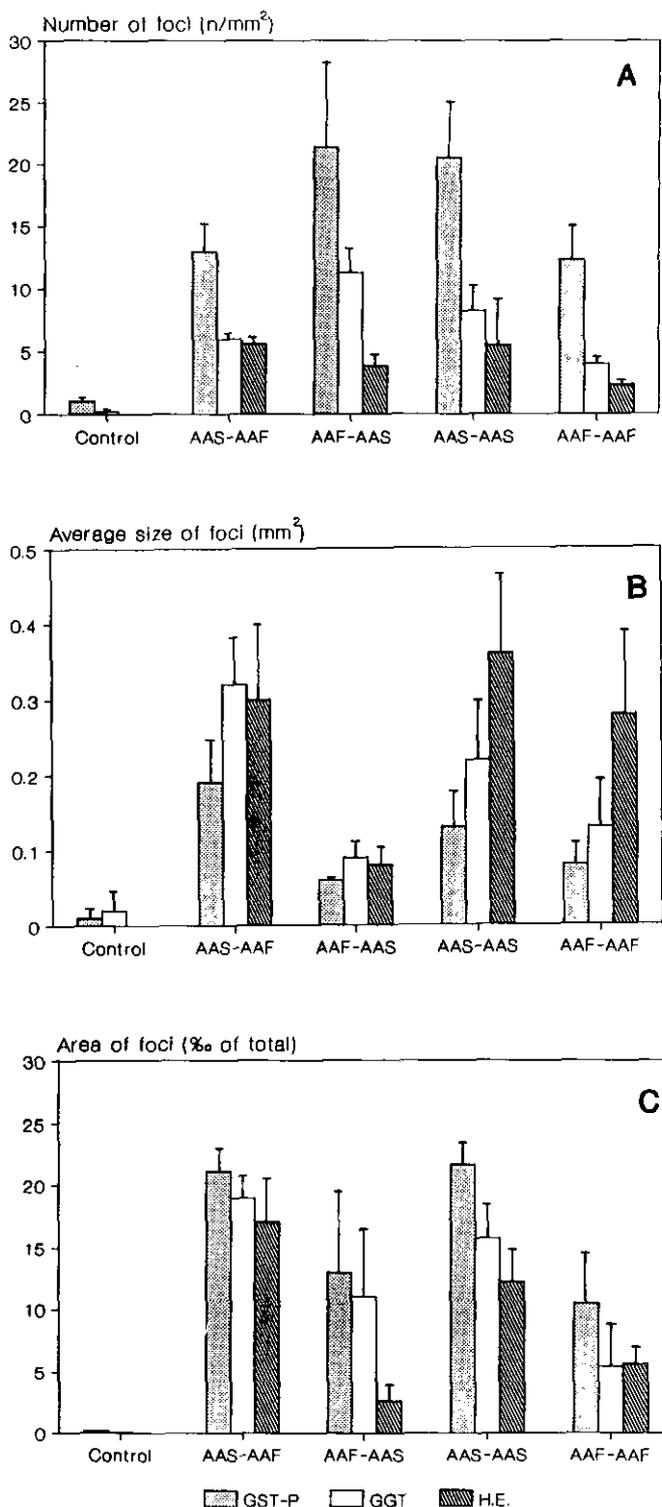


FIGURE 2. Comparison of the number (A), average size (B), and total area (C) of foci in rat liver after oral administration of the initiator combination indicated to adult animals after partial hepatectomy and 26 weeks of phenobarbital treatment.

vanced stages, so that the average size of foci is greater in stained sections. These differences are partly compensated when looking at the total area. One overall

Table 3. Generation of enzyme-altered liver foci in newborn male Wistar rats.^a

| Initiation day | | Dose, μmole/kg | | Promotion, mg/L in drinking water 500 mg/L | Total area, per thousand | | | | |
|----------------|-----------|-------------------|---------|--|--------------------------|---------|---------|------|-----|
| | | | | | γ-GT | | GST-P | | |
| 6, 8 | 12, 14 | | | | | Week 10 | Week 20 | | |
| Control | | — | — | PB* | | 0.04 | 0.06 | 0.06 | 0.3 |
| AAS | AAS | 100 | 100 | PB | | 4.2 | 4.6 | 6.1 | 6.0 |
| AAS | AAF | 100 | 250 | PB | | 0.5 | 2.3 | 0.7 | 3.4 |
| AAS + AAF | AAS + AAF | 100/250 | 100/250 | PB | | 0.8 | 3.0 | 1.3 | 3.4 |
| AAF | AAF | 250 | 250 | PB | | 0.4 | 1.3 | 0.7 | 1.6 |

*PB, phenobarbital

result is that a 5-fold total dose of AAF produces a smaller effect than AAS, and this seems to be due primarily to the greater number of foci produced by AAS.

In the combination groups, the number of foci seems to be determined by the second combination partner, i.e., the figures for AAS-AAF are similar to those of AAF-AAF, and the figures of AAF-AAS are similar to those of AAS-AAS (Fig. 2). The average size is not significantly different in the two control groups, but clearly differs between the two combination groups, foci in the AAS-AAF group being significantly larger. If the total area covered by foci is taken as the overall effect, then the combination AAS-AAF is more efficient than expected from the addition of half the effects in the two controls.

Synergism in a Simplified Initiation-Promotion Protocol

The use of newborn animals (Fig. 1B) (Table 3) had several advantages. Partial hepatectomy could be avoided because the livers are subject to an endogenous proliferation stimulus during the first 3 weeks of life. In addition, only two doses of each initiator were required, which comes closer to single-dose application. In this experiment AAS was again a better initiator than AAF. The initiating effects of the two chemicals, however, were simply additive. Promoting properties of AAF during initiation were not apparent. The simplified initiation-promotion model using newborn animals, therefore, appears to be particularly suitable for testing synergism of initiators. An additional problem was addressed: Is there a difference if the initiators are administered as a mixture rather than sequentially? This does not seem to be the case (Table 3).

Conclusions

The results further support the following notions: AAS is a strong initiator in rat liver. AAF has both initiating and promoting properties. The initiating effects of these two chemicals generating different types of DNA adducts are, at least, additive. Promoting, presumably nongenotoxic effects, rather than initiating effects, determine the tumor development in tissues

hitherto called target tissues and the rate of tumor growth.

Synergistic effects of genotoxic chemicals may be more important in the real-life situation than hitherto accepted. In addition, carcinogenicity testing in animals does not sufficiently reveal the role of secondary effects. This adds to the problems encountered in quantitative risk assessment for humans.

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