Induction and Inhibition of Tumor Progression (43511B)

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Carcinogenesis has been demonstrated by experimental and epidemiologic studies to be a multistage process composed of three major stages, initiation, promotion, and progression (1). The progression stage of this process encompasses the changes by which a tumor develops from a contained, differentiated lesion of uncoordinated growth into a rapidly growing, invasive lesion that can spread to other areas of the body, i.e., from a benign to a malignant tumor. Progression is of great therapeutic relevance because the process involves a change from a lesion that is easily treated surgically to one requiring extensive therapy with less favorable outcome.

Tumor Progression in Mouse Skin

The mouse skin model of carcinogenesis has served a primary role in the current understanding of the mechanisms of progression and antiprogression. This review will focus largely on this system and on the initial invasive stages of tumor progression associated with the conversion from a papilloma to a carcinoma.

The mouse skin model is especially adaptable to the study of tumor progression. One large benefit of this model is the production of tumors on the exterior of the animals. This allows ready monitoring of changes in the occurrence, size, and type of tumors. A typical treatment protocol is as follows. Animals are first treated with an initiating agent, such as 7,12-dimethylbenz(*a*)anthracene (DMBA), at Time 0 and after a 2-week period, biweekly treatment with a promoting agent (typically 12-*O*-tetradecanoylphorbol-13-acetate [TPA]) is begun. Using this protocol, papillomas are first visible at 6–8 weeks and reach a plateau at about 20 weeks. Carcinomas are initially seen at approximately 30 weeks and reach a maximum number at about 50 weeks.

Carcinogens are used to induce the initiation stage of carcinogenesis in mouse skin. These compounds will, by definition, cause the formation of tumors in the absence of other treatments, provided the dose or the frequency of administration is great enough. However, doses used in multistage protocols are subtumorigenic and will not produce tumors in the animal's lifetime in the absence of other treatments. These agents are typically genotoxic and induce changes that are irreversible, lasting for the lifetime of the animal. The relevant genotoxicity is thought to result in the activation or inactivation of cancer-related genes (2).

Promoting agents, in contrast to initiating agents, are not carcinogenic and produce few tumors in the absence of other treatments. The effects of promoting agents are also reversible and these compounds must be given at the proper dose and frequency of administration to be effective. Numerous activities in the skin have been described for this class of compounds; however, the ability to produce a sustained hyperplasia correlates best with tumor promotion (3). Related changes after treatment with TPA are exemplary. Multiple TPA treatments lead to a 5-fold increase in [^{3}H] thymidine incorporation in the basal layer of epidermis and an increase in skin thickness approximately four to five times normal (4).

The simplest model for the formation of papillomas after initiation and promotion is as follows. The initiating agent leads to an oncogenic mutation in a limited number of cells within the skin that remain in a repressed state due to interactions with surrounding normal cells. Treatment with promoting agents induces cellular proliferation and hyperplasia, negating the repression of the initiated cells by surrounding normal cells. The generation of such an environment allows expansion of the initiated cell population ultimately leading to the formation of a papilloma.

Carcinomas are observed after approximately 30 weeks of treatment and develop in the vast majority of cases from existing papillomas (5). The incidence of carcinomas is thus related to the number of papillomas

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P.S.E.B.M. 1993, Vol 202: 9–15

0037-9727/93/2021-0009$3.00/0

formed; however, there is an apparent maximum tumor mass that an animal can support. This sets a limit of three to four carcinomas per animal. The ratio of papillomas to carcinomas or percentage of conversion can range from 5% in protocols that have a maximal papilloma response to 40% where fewer papillomas are produced (6).

Agents and Mechanisms that Increase Tumor Progression

Repeated treatment of animals with a carcinogen, in complete carcinogenesis protocols, produces fewer papillomas and a larger number of carcinomas than the multistage treatment protocols described above (7). Carcinomas produced in this manner arise at earlier time points and, in some cases, develop directly on the epidermis in the absence of visible papilloma formation. These responses suggest that carcinogens are able to generate the necessary lesions for the formation of both papillomas and carcinomas. To address this issue, our group (8) and others (9, 10) designed experiments to determine whether the treatment of papillomas with carcinogens could lead to an increase in the rate of formation of carcinomas from papillomas. This was indeed the case, and, as shown in Figure 1, treatment of papilloma-bearing skin with the direct-acting carcinogens ethylnitrosourea or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) resulted in an earlier appearance of carcinomas and an increase in the percentage of mice with carcinomas.

In these experiments (Fig. 1), three groups of animals were initiated with DMBA (10 nmol) and treated with TPA (1 μg) biweekly for 20 weeks until the formation of papillomas had stabilized. The animals were then divided into three groups adjusted for papilloma number. TPA treatment was then discontinued for two of the groups and these animals were treated with 10 μmol of ethylnitrosourea or 1 μmol of MNNG twice weekly for 2 weeks; the third (control) group received TPA. After these treatments, the animals received biweekly TPA treatment until Week 40. As stated above, the carcinogen-treated animals formed carcinomas earlier and in larger numbers. It should also be noted that there was no increase in the cumulative number of papillomas in these groups during the progression stage. Thus, the effect of the ethylnitrosourea or the MNNG under these conditions was upon existing papillomas rather than the generation of new tumors.

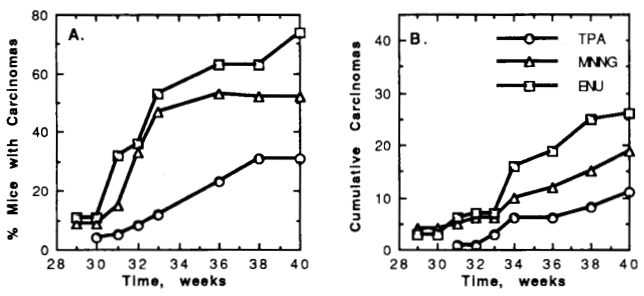

Figure 1. (A) The percentage of mice with carcinomas and (B) the cumulative number of carcinomas per group for mice treated during the progression stage of carcinogenesis with TPA, MNNG, or ethylnitrosourea (ENU).

Agents with tumor-progressing activity are listed in Table I. Some of the carcinogen dosage regimes in these studies rival complete carcinogenesis protocols. Carcinogen treatments were carried out for periods as long as 30 weeks. The increases in carcinoma formation in these studies were clearly greater than those for controls; however, mechanistic evaluation of such regimens is difficult.

Repeated treatment with carcinogens does not appear to be required to increase tumor progression, as certain agents have demonstrated high activity after a single treatment. Studies in our laboratory showed that a single noncarcinogenic dose (200 nmol) of benzo (*a*)pyrene diol epoxide lead to a 3-fold increase in the cumulative number of carcinomas formed. Hennings and co-workers (11) demonstrated that a single treatment of cisplatin, at a noncarcinogenic dose, also resulted in an increase in tumor progression. Although the dose-response relationships for tumor progression have not been developed, these results suggest that benzo(*a*)pyrene diol epoxide and cisplatin induce specific genetic changes necessary for tumor progression. These agents may thus aid in the identification of specific progression-associated lesions.

Expanding the model presented earlier for papilloma formation, these results support the involvement of a genetic lesion(s), in addition to those associated with initiation, in the progression of papillomas to carcinomas. Such a lesion(s) would occur at a low rate in papillomas, leading to the low number of carcinomas formed from papillomas in the absence of progressor treatment. However, treatment of papillomas with carcinogens leads directly or indirectly to an increase in the occurrence of this lesion(s) and, hence, to carcinomas. This model predicts that carcinoma formation requires at least two oncogenic mutations and, indeed, transfection studies have demonstrated that expression of two activated oncogenes can induce malignant progression (12).

There is a possible difference in the requirements for the generation of an initiating lesion and a progressing lesion. This is exemplified by benzoyl peroxide and hydrogen peroxide. These agents, although genotoxic, are not carcinogenic agents, yet they are able to induce tumor progression. Studies in our laboratory have examined the tumorigenic activity of benzoyl peroxide (13, 14). Benzoyl peroxide was found to have no activity as a complete carcinogen or tumor initiator; however, this agent had good activity as a tumor promoter. In these studies, we observed an inordinate number of

Table I. Agents that Increase Tumor Progression in Mouse Skin

Agent	Treatment	Ref.
Carcinogens		
Benzo(*a*)pyrene diol epoxide	200 nmol, topical, once	UO[a]
Cisplatin	335 nmol, ip, once	11
MNNG	1 μmol, topical, biweekly, 4x	8
MNNG	815 nmol, topical, weekly, 30x	9, 10
Urethane	225 μmol, ip, weekly, 30x	9, 10
Ethylnitrosourea	10 μmol, topical, biweekly, 4x	8
4-Nitroquinoline-*N*-oxide	1.5 μmol, topical, weekly, 30x	9, 10
Free radical generators		
Benzoyl peroxide	85 μmol, topical, biweekly, 30x	14, 15
Hydrogen peroxide	15% Soln, topical, biweekly, 12x	15
Other		
Acetic acid	670 μmol, topical, biweekly, 30x	22
Diethyl maleate	30 μmol, topical, biweekly, 30x	20

[a] UO, unpublished observations.

carcinomas. In subsequent investigations, benzoyl peroxide was examined as a progressing agent and was found to produce more than a 3-fold increase in the cumulative formation of carcinomas. Subsequent studies also demonstrated that hydrogen peroxide has similar activity (15). The mechanism for enhanced progression by these peroxides is currently unclear. The peroxides are free radical generators and have been shown to produce single-strand DNA breaks (16, 17) and DNA-protein cross-links (18). Such genetic lesions may be sufficient to cause tumor progression. Because the peroxides are not carcinogenic, these effects must not be adequate for initiation or complete carcinogenesis. This difference may be due to a progressive increase in genetic instability observed during tumor promotion (19) that may have an additive effect with that of the peroxides.

Other investigations in our laboratory suggest a role for free radicals in tumor progression. The tripeptide glutathione (GSH) acts as one of the principle molecules of cellular defense against reactive oxygen species. To evaluate the role of oxidative damage in tumor progression, we designed studies to both increase (discussed later) and decrease the levels of GSH in papilloma-bearing skin. Diethyl maleate has been demonstrated to decrease the GSH content of mouse skin after topical treatment (20). Biweekly topical treatment of papilloma-bearing skin with 30 μmol of diethylmaleate led to a 1.6-fold increase in the percentage of mice that developed carcinomas (Fig. 2B). Although other interpretations of this experiment will be discussed later, GSH can play a role in the prevention of tumor progression.

Another potential mechanism for the tumor-progressing activity of the peroxides invokes selective toxicity of noninitiated epidermal cells in response to these compounds. Keratinocyte cell lines developed from initiated skin or papillomas have been found to be

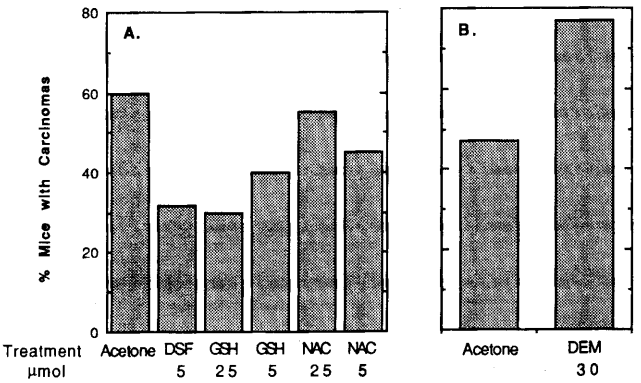

Figure 2. The percentage of mice with carcinomas after treatment during the progression stage of carcinogenesis with (A) disulfiram (DSF), GSH, or *N*-acetyl cysteine (NAC) or (B) diethylmaleate (DEM).

resistant to DNA strand breakage and cytotoxicity by benzoyl peroxide and, within a group of 10 cell lines, there was an inverse correlation between malignant behavior and DNA strand breakage by benzoyl peroxide (21). The resulting selective toxicity could lead to elimination of cells without malignant potential, thereby allowing malignant cells the space and nutrients for expansion.

Studies in our laboratory examining acetic acid as a tumor-progressing agent also support selective toxicity as a potential mechanism in tumor progression. Treatment of papilloma-bearing mice biweekly with acetic acid (667 μmol) led to a 2-fold increase in both the percentage of animals with carcinomas and the cumulative number of carcinomas formed (22). The dosage used here may well have had toxic effects. Earlier studies had demonstrated that acetic acid was a very weak tumor promoter (23). Acetic acid produced an increase in epidermal DNA synthesis, which is typical of tumor promoters; however, the time period before maximal DNA synthesis increased with the dose of acetic acid utilized. This suggests that the increase in

DNA synthesis was reparative and the result of a progressive increase in toxicity. Indeed, overt toxicity was observed at the highest dose of acetic acid (1000 mmol). Thus, toxicity may well be occurring at the dose utilized in the above studies and may play a role in the increased formation of carcinomas. A second interpretation is that since increased DNA synthesis has been associated with increases in mutation rates (24), the increased DNA synthesis, dependent or independent of the toxicity observed here, could potentially lead to malignant lesions.

In addition to these studies in mouse skin, studies in other tumor systems indicate tumor progression occurs as the result of cumulative genetic lesions. Examples of such systems would include the colon (25), lung, and breast (2). Accordingly, intervention to deter mutagenesis will have good potential for the prevention of tumor progression.

Inhibition of Tumor Progression

Oxidative stress has a potentially important role in tumor progression, as discussed above. Addressing this mechanism, we have examined free radical scavengers and antioxidants as inhibitors of the tumor progression process. The use of such compounds is not without precedence, as they have activity as inhibitors of tumor initiation and tumor promotion (26). Table II lists the agents examined, dosages utilized, and the resulting activities. For these studies, animals were initiated with DMBA and promoted with TPA for 16 weeks, at which point, TPA treatment was terminated. After 4 weeks with no treatment, the animals were assigned to groups adjusted for papilloma number, and papilloma-bearing skin was treated biweekly with the putative progression inhibitors for the remaining 30 weeks of the study. Only reduced glutathione and disulfiram were shown to have significant activities as suppressors of tumor progression. Pharmacokinetic effects could have contributed to these results because only a single dose, which inhibited tumor promotion, was examined.

In agreement with the studies using diethyl maleate, a 25-μmol dose of reduced glutathione led to a 50% reduction in the formation of carcinomas, as shown in Figure 2A. GSH treatment of epidermal cells has been shown to increase intracellular GSH levels and this has an inhibitory effect on tumor progression. GSH and the GSH transferases are considered to be important components in the prevention of mutagenesis (27) and may possibly be involved in this effect. In interesting contrast, a progressive increase in intracellular GSH levels is observed as different types of tumors, including papillomas, progress to malignancy (28). As will be discussed in detail below, recent studies in our laboratory suggest that GSH may have both inhibitory and stimulatory actions in tumor progression.

Disulfiram had moderate antiprogression activity, reducing by approximately 50% the number of animals with carcinomas. Disulfiram is also good inhibitor of tumor initiation in chemical carcinogenesis studies. Modulations of several pathways by disulfiram have been described that may be relevant to this effect. These would include inhibition of oxidative metabolism (29), direct interaction with electrophiles (30), increases in cellular thiol levels, and induction of GSH transferase (31).

It would have been reasonable to predict that a cysteine source such as *N*-acetylcysteine would have activity similar to GSH, as cysteine pools are generally considered to be rate limiting for GSH synthesis (32). However, studies in our laboratory observed no significant inhibition of tumor progression with *N*-acetylcysteine. It is possible that the compound was not absorbed through the skin or the dosages were insufficient to increase epidermal cysteine pools.

Unexpectedly, butylated hydroxyanisole (BHA) acted as a tumor-progressing agent in these studies. BHA treatment led to a significant increase in the percentage of papillomas that became carcinomas (11% vs 7.8% for the carrier-treated animals). This result was surprising because BHA has inhibitory activity against both tumor initiation and promotion and has been shown to increase GSH transferase activity (24). However, in the forestomach model of carcinogenesis, BHA does act as a tumor promoter (33). These results emphasize that understanding of these processes is incom-

Table II. Agents Examined as Inhibitors of Tumor Progression

Agent	Dose[a]	Activity[b]
Reduced glutathione	5 μmol, 25 μmol	Moderate
Disulfiram	5 μmol	Moderate
Butylated hydroxyanisole	50 μmol	+
Vitamin E	5 μmol	—
Copper(II)(3,5-diisopropylsalicylate)$_2$	5 μmol	—
Sodium benzoate	50 μmol	—
N-acetylcysteine	5 μmol, 25 μmol	—

[a] All treatments were topical and biweekly.
[b] —, No activity observed; +, tumor-progressing activity.

plete. It can be expected that chemical inhibition of tumor progression will follow the general principles found for the inhibition of the other stages of carcinogenesis in which multiple variables, including dosage, time, and tissue examined, determine the efficacy of inhibition.

Alteration of GSH Levels by γ-Glutamyltranspeptidase Overexpression

Recently, we studied the effects of the overexpression of γ-glutamyltranspeptidase (GGT) in an epidermal cell line. GGT is thought to function physiologically in the maintenance of glutathione homeostasis (32). The reaction catalyzed by GGT involves the transfer of γ-glutamyl groups to acceptor peptides, amino acids, or water (34). GSH is poorly transported into cells and GGT catalysis of GSH makes available the amino acid constituents for intracellular transport and resynthesis of GSH. GGT is one of only two enzymes that have demonstrated the ability to cleave GSH (35).

Studies both in our laboratory and others have established that GGT is a marker protein for tumor progression in mouse skin (36, 37). With the exception of the hair follicle, GGT is not histologically detectable in normal adult epidermis and it is also not observed in early papillomas. However, in late papillomas and in carcinomas, GGT becomes histologically detectable. For evaluation of the role of GGT in causing tumor progression, we transfected an epidermal cell line, which does not grow subcutaneously and which produces papillomas in skin grafts (38), with a functional cDNA for human GGT (the generous gift of Dr. Henry C. Pitot, University of Wisconsin, McArdle Laboratory, Madison, WI).

GGT overexpression had no apparent effect on the *in vitro* growth of epidermal cells. There was no significant difference in the saturation density or the requirement of serum for growth between GGT and vector transfectants. In addition, no distinguishable morphologic differences between the cell types was observed.

Subcutaneous growth in nude mice is a standard assay for potential malignancy of cell lines and was used to evaluate the GGT and vector-transfected clones. As shown in Table III, the GGT transfectants produced tumors at all sites where they were injected. In contrast, injection of four different vector transfectants produced tumors in only 24% of the sites injected. The simplest interpretation of these results is that GGT provided the cells with a growth advantage in a subcutaneous environment.

Clones were also grafted into the skin of nude mice to evaluate their tumorigenic growth in an environment closer to their normal cellular milieu. The GGT transfectants demonstrated a growth advantage in grafts, producing significantly larger tumors with an average mass that was 3-fold that of the vector-transfected

Table III. Subcutaneous Injection of Cells that Overexpress γ-Glutamyltranspeptidase into Nude Mice

Cell line	Sites injected (*n*)	Tumors (*n*)	Sites with tumors (%)
γ-GT clones			
E1E9	6	6	100
E1E4	5	5	100
H3H4	4	4	100
Total γ-GT	15	15	100
Vector clones			
F10	6	1	17
E5	4	1	25
D12	3	0	0
D11	8	3	38
Total vector	21	5	24

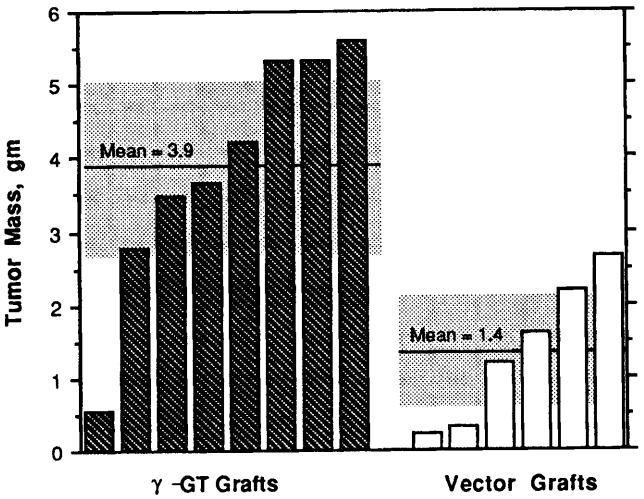

Figure 3. Tumor mass after grafting of γ-glutamyltranspeptidase or vector-transfected cells into nude mice. Gray areas represent SE (means are different, $P < 0.05$).

clones (Fig. 3). However, histologically, no difference was observed between the GGT and the vector transfectant-derived tumors; both cell types produced masses resembling epidermal inclusion cysts, with occasional areas identifiable as squamous cell carcinoma. In summary, overexpression of GGT provided these cells with a definite growth advantage subcutaneously and in grafts; however, the role for GGT in the progression of naturally occurring tumors remains unclear.

GSH levels were also examined in experimental tumors produced after grafting and in the normal skin of the graft recipients. GSH levels in GGT transfectant-derived tumors were approximately one third of that in the vector transfectant-derived tumors and about one half of that in normal skin (unpublished data). The GSH levels of the cells in culture were also examined; GSH in the GGT transfectants was less than half that of control cells. The low levels of GSH in the GGT-

transfected cells and tumors were surprising for the following reasons: (i) this enzyme is thought to play a positive role in GSH homeostasis (32); (ii) intracellular GSH levels progressively increase during carcinogenesis and are highest in carcinomas (28); and (iii) GSH has inhibitory activity toward tumor progression (20).

However, evidence also exists that suggests that GSH may function as a double-edged sword—both inhibiting and, through its catabolic product, cysteinylglycine, stimulating tumor progression. Preliminary studies with these cells indicate an altered localization of the overexpressed GGT in the interior rather than on the external surface of these cells. GGT, in this localization, would indeed catalyze the breakdown of intracellular GSH. Furthermore, catalysis of GSH may possibly lead to increased oxidative stress within these cells. GSH, in the presence of GGT, has been demonstrated to give a positive Ames test in a Salmonella strain known to be susceptible to oxidative damage (39). This effect could also be produced by a catabolic product of GGT and GSH, cysteinylglycine, and the requirements and inhibitors for this effect also support a role for oxidative damage as the proximate mechanism (40). In further support, the progressing agent diethyl maleate has been shown to deplete cellular GSH. Although it has the ability to interact directly with GSH, diethyl maleate also stimulates the activity of GGT (41). Such an effect would also lead to an increase in cysteinylglycine with associated oxidative damage. We are currently examining this mechanism as a source of the tumorigenic functioning of GGT.

In conclusion, the process of tumor progression has not been thoroughly studied and, because this is a clinically relevant stage of carcinogenesis, extensive examination is warranted. Tumor progression has been well aligned with mutagenic changes. Further identification of these lesions and their biochemical function should take priority in future studies. With a greater foundation of understanding of the events in this process, we can expect the development of compounds that are able to inhibit or bypass the function of these molecular processes. Such agents hold great potential to inhibit and possibly reverse the tumor progression process.

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