Response of Mouse Skin Tumors to Doxorubicin Is Dependent on Carcinogen Exposure

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ABSTRACT

To investigate the role of carcinogenesis in determining the response of tumors to anticancer drugs, we have used the in vivo model of multistage carcinogenesis of the mouse skin. Mice were initiated with Harvey murine sarcoma virus or single and repeated applications of dimethylbenzanthracene (DMBA). The papillomas which developed as a result of these initiation protocols were monitored quantitatively for their response to the anticancer drug doxorubicin. A single dose of 10 mg/kg doxorubicin is relatively inefficient at reducing the frequency of papillomas arising as a result of either single or repeated applications of the chemical DMBA. However, virally initiated papillomas are sensitive to the single 10-mg/kg dose of doxorubicin and are reduced in frequency by greater than 80%. Repeat treatment with four doses of 5 mg/kg doxorubicin over a 4-week period also reveals differences in the responses of the papillomas to doxorubicin. As with the single dose of doxorubicin, papillomas initiated with multiple applications of DMBA showed only a limited response to four 5-mg/kg doses of doxorubicin. In comparison both the virally initiated and the single DMBA initiated papillomas responded to the four doses of doxorubicin and are reduced in frequency by about 80%. These data show that the response of papillomas to doxorubicin is related to the initiating event. Papillomas derived by viral initiation are most sensitive to doxorubicin while increasing the level of exposure to the chemical carcinogen DMBA increases the proportion of papillomas which do not respond to treatment with doxorubicin. There was no obvious relationship between the method of initiation or the treatment of the mice with doxorubicin and the levels of P-glycoprotein expression observed in the papillomas. All the papillomas expressed detectable levels of P-glycoprotein approaching that of the multidrug resistant cell line, CHRC5.

INTRODUCTION

The incidence of many types of cancer in human populations is associated with exposure to environmental carcinogens (1). However, it is studies of chemical carcinogenesis in animal model systems which most clearly describe cancer development as a multistep process and systematically examine the molecular events associated with carcinogen exposure (2–5). Although the relationship between carcinogen exposure and tumor development is extensively studied (4, 5), the relationship among carcinogen exposure, tumor development, and the response of tumors to anticancer drugs is poorly understood. The resistance of tumors in vivo to anticancer drugs is a complex problem involving tumor, host, and drug interactions (6). Resistance may be linked to inter- and intratumor heterogeneity in cellular responses to drug exposure such as repair of drug induced damage, detoxification enzymes, or drug efflux (7, 8). Success of chemotherapy may also be limited by normal tissue toxicity and pharmacokinetics (9).

There are a number of in vivo models relating to carcinogenesis (2, 5) but at present only rat hepatocarcinogenesis has been studied in terms of tumor resistance to toxic agents (4, 10). Hepatocarcinogenesis administration in rats causes preneoplastic and neoplastic nodules in the liver (10). These nodules are resistant to the cytotoxic effects of some hepatocarcinogens which may be attributed in part to altered patterns of carcinogen metabolism in comparison to normal hepatic tissue (11–15). Thus, it has been suggested that the clinical resistance of tumors to anticancer drugs may be related to the mechanisms by which carcinogen exposed cells survive the toxic effects of the carcinogen (16). Indeed carcinogen exposed hepatocytes are more resistant to anticancer drugs such as doxorubicin than normal hepatocytes (12, 16). The expression of genes associated with carcinogen metabolism in liver after carcinogen exposure is extensively documented (17). Interestingly chemical carcinogenesis of liver also induces expression of a multidrug resistance mdr gene encoding a M, 175,000 P-glycoprotein (18–20) involved in active efflux of many common anticancer drugs such as doxorubicin out of multidrug resistant cell lines (21, 22).

In this study we have used the well characterized model of multistage carcinogenesis of the mouse skin (2, 5, 23) to analyze the response of tumors in vivo to the chemotherapeutic drug doxorubicin (24). Despite the widespread use of doxorubicin as a chemotherapeutic drug, mechanisms which underlie its toxicity are poorly understood. Doxorubicin can undergo metabolism to yield superoxide radicles which may cause oxidative damage to both DNA and cell membranes (25). Doxorubicin also interacts with topoisomerase II, a nuclear enzyme involved with DNA metabolism (26). The multistage model of carcinogenesis of the mouse skin is described by an initiation and a promotion stage (23). Initiation of the skin with a low dose of carcinogen introduces DNA lesions into a population of cells (2, 23). Initiated cells retain a nontumorigenic phenotype until an appropriate local environment is created by repeated application of a tumor promoter (27, 28). During promotion the initiated cells proliferate and form papillomas (23, 28). A proportion of the papillomas will progress to malignant carcinomas, thus completing the carcinogenic process over a period of months (23). Over 90% of papillomas initiated with the polycyclic hydrocarbon DMBA1 have an activating A to T transversion in codon 61 of the Harvey ras (Ha-ras) oncogene (3). Whether the mutations observed which relate to carcinogenesis are the sole effects of exposure to the initiators is unknown. When a virus containing the Ha-ras oncogene is applied directly onto the mouse skin, tumors are also induced (29), providing further evidence that ras gene mutation is an early event in this carcinogenesis system (3, 23). Multistage carcinogenesis of the mouse skin is therefore ideally suited to study the relationship between carcinogen exposure and drug resistance in tumors. We show that the chemically and virally induced papillomas respond to the chemotherapeutic agent doxorubicin and that this response is related to the initiator used.

1 The abbreviations used are: DMBA, 7,12-dimethylbenz(a)anthracene; TPA, 12-O-tetradecanoylphorbol-13-acetate.
MATERIALS AND METHODS

Virus Preparation

HT3 cells which release Harvey murine sarcoma virus and Friend murine leukemia helper virus were obtained from Dr. A. Balmain (Beatson Institute, Glasgow, United Kingdom). Virus containing supernatant from the virus producer cell line HT3 was concentrated and stocks were titered on normal rat kidney cells (29).

Skin Tumor Induction

Female NIH/SWISS mice ages 6–8 weeks were used in all experiments. Mice were obtained from Olac.

Viral Initiation (29). Mice were shaved at least 2 days before initiation. One day prior to initiation animals were pretreated with topical administration of TPA (200 µl of 10^-4 M). Harvey murine sarcoma virus was used at a concentration of 1 x 10^6 focus forming units/ml. Approximately 200 µl of viral supernatant containing 2 µg/ml Polybrene were applied to the backs of anesthetized mice by scarification using a 25-gauge needle attached to a 1-ml syringe. One week after initiation the animals were treated twice weekly for 4 weeks with TPA (200 µl of 10^-4 M solution in acetone).

Chemical Initiation (3). Mice were shaved at least 2 days before initiation: (a) a single application of 25 µg DMBA in 200 µl of acetone was made to the skin of the back of each mouse. After 1 week, twice weekly applications of 200 µl 10^-4 M TPA were begun and continued for 8 weeks; (b) exposure of the mouse skin to multiple doses of DMBA was carried out by applying DMBA (25 µg/200 µl acetone) twice weekly for 7 weeks, followed by twice weekly applications of TPA (200 µl of 10^-4 M TPA) for 4 weeks.

Representative papillomas from each initiation group, virus, single DMBA, and multiple DMBA were removed for histological examination. Dr. H. Thompson (Pathology Department, Glasgow Veterinary School) confirmed that all the papillomas were squamous cell papillomas derived from normal stratum spinosum and that histologically the papillomas from each initiation group were similar (data not shown).

Drug Administration

Prior to drug administration mice were individually identified with an ear tag and the frequency of papillomas per animal was documented. Papilloma frequency was recorded as the total number of papillomas on each mouse and also the frequency of these papillomas greater than 2 mm in two dimensions. The mice were then split into groups ensuring that as far as possible each group within an experiment contained approximately the same total number of mice, total numbers of papillomas, and total numbers of papillomas >2 mm.

At least 1 week after the final application of TPA, treatment groups of mice of 10 to 25 mice received i.v. injection of doxorubicin via the tail vein. Single dose groups received one injection of 10 mg/kg doxorubicin whereas multiple dose groups received 4 injections, 1/week, of 5 mg/kg doxorubicin. These drug doses did not result in animal deaths over the time periods of the experiments. The response of papillomas to doxorubicin was monitored for up to 8 weeks by counting the papillomas and recording this as total papillomas per mouse and the number of these >2 mm in two dimensions. Papillomas were counted while the mice were anesthetized.

Estimation of Papilloma Growth Rate

Papillomas are detectable at cessation of TPA treatment as small raised lesions on the skin. In this study the total number of such lesions was counted and recorded as the total number of papillomas per mouse. Any papillomas which had attained 2 mm in two dimensions were categorized as a subset of the total papillomas per mouse. The percentage of the total papillomas >2 mm in two dimensions recorded over time gives an estimation for the rate of papilloma growth in any group. Fig. 4 shows rates of papilloma growth for the control groups in each experiment. The regression coefficients for the control groups in the single DMBA, multiple DMBA, and viral initiation protocols are 9.90, 7.46, and 17.7, respectively. The data used to calculate the coefficients were weeks 1 to 7 for the DMBA experiments and weeks 1 to 4 for the viral experiment as these are the linear parts of the graphs.

Chemicals

TPA purchased from the Sigma Chemical Company was dissolved in acetone and applied as 200 µl of a 10^-4 M solution. DMBA, also purchased from Sigma, was dissolved in acetone and applied as 25 µg in 200 µl. Doxorubicin was purchased from Farmitalia Carlo Erba, Ltd., and dissolved in water at 2 mg/ml.

Isolation of Proteins and Protein Blot Analysis

Papillomas removed for molecular analysis were frozen immediately in liquid nitrogen and stored at -70°C. Frozen samples were pulverized while still frozen in a microdisembranator II (Braun, Federal Republic of Germany) and proteins were solubilized in 0.1 M Tris, pH 8-10% glycerol-0.5% Nonidet P-40. Protease inhibitors phenylmethylsulfonyl fluoride (100 µg/ml), pepstatin (1 µg/ml), and aprotinin (2 µg/ml) were included. Cell lines A2780 (30), AXL81, and CHRC5 (22, 31) were lysed directly in the above buffer. Solubilized proteins were spun for 2 min in a microfuge at 0°C to remove insoluble particles and 50 µg were run per lane.

Electrophoresis was performed by a slight modification of the method of Fairbanks et al. (32, 33), and as described by the distributors of the C219 antibody, Centocor Belgium. Modifications include addition of 2% sodium dodecyl sulfate and 4.5 M urea to the sample buffer. Samples were not boiled prior to loading. 5% sodium dodecyl sulfate-polyacrylamide gels were run with the inclusion of urea to a final concentration of 9 M. Proteins were transferred to nitrocellulose by semidry electrobolting (34) and the membrane was blocked for 2 h at room temperature in 10 mM Tris-HCl, pH 7.4-0.9% NaCl-3% bovine serum albumin-0.05% Tween 20. C219 antibody which recognizes the P-glycoprotein family of membrane proteins (35) was purchased from Centocor Belgium and CIS (UK) Ltd. The C219 antibody was diluted in the above solution to give a final concentration of 1 µg/ml and added to the blots overnight at room temperature. Protein-antibody complexes were visualized by enhanced chemiluminescence as recommended by the manufacturers, Amersham International, Plc., (United Kingdom). Protein dot blots were treated identically to Western blots after transfer to nitrocellulose. The dilutions were 1:10.

RESULTS

Response of Papillomas Initiated with a Single Dose of DMBA to Doxorubicin. Mice initiated with 25 µg of DMBA were split into 3 groups. The single dose group was given injections of one dose of 10 mg/kg doxorubicin while the multiple dose group received four injections over 4 weeks of 5 mg/kg doxorubicin (weeks 1 to 4 in Fig. 1). As can be seen in Fig. 1A, the total number of papillomas in the untreated control group remains constant over the 18-week period of the experiment. Over this period of time the frequency of papillomas >2 mm rises and appears to plateau at around week 10 (Fig. 1B). Administration of a single 10-mg/kg dose of doxorubicin reduces the frequency of papillomas by 12% as calculated from week 10 onward (Fig. 1). However, multiple administration of 5 mg/kg doxorubicin over 4 weeks reduces the total number of papillomas by 75% by week 6 (Fig. 1A), a reduction which is also mirrored in the analysis of papillomas greater than 2 mm (Fig. 1B). Fig. 2 relates the frequency of papillomas >2 mm to the total number of papillomas of all sizes over time, thus deriving a rate of growth for the papillomas in each group. The regression coefficients for the rates of growth in the control, single dose, and multiple dose groups as measured for weeks 1 to 7 are 9.90, 6.75, and 0.93, respectively. From Fig. 2 it can be seen that the papillomas in the single and multiple dose.
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Fig. 1. Response to doxorubicin of papillomas initiated with a single application of 25 μg DMBA. The response to doxorubicin of total papillomas (A) or papillomas >2 mm in two dimensions (B) is shown over time in weeks. The average papilloma number per mouse is the average of 12 mice/group counted at the time shown. Standard error (bars) shown for B only. O, average number of papillomas per mouse in the absence of treatment with doxorubicin; A, average number of papillomas per mouse after a single tail vein injection of 10 mg/kg doxorubicin in week 1; •, average number of papillomas per mouse after 4 tail vein injections of 5 mg/kg doxorubicin on weeks 1 to 4.

Fig. 2. Growth rates of papillomas initiated by a single 25-μg application of DMBA. The percentage of total papillomas >2 mm in two dimensions in any experimental group plotted over time is used to calculate the average growth rate for papillomas. O, growth rate of papillomas in the absence of treatment with doxorubicin; Δ, average number of papillomas per mouse after a single tail vein injection of 10 mg/kg doxorubicin in week 1; •, growth rate of papillomas after 4 tail vein injections of 5 mg/kg doxorubicin on weeks 1 to 4.

Gruppen initially have slower growth rate.

Response of Papillomas Arising from Skin Treated with Multiple Doses of DMBA. In order to test whether the dose of carcinogen encountered by the skin affected the subsequent response of papillomas to doxorubicin, mice were treated with 14 applications of 25 μg of DMBA. The animals were split into 3 groups and treated with doxorubicin as described for a single initiating dose of DMBA. Fig. 3 shows that both the single and multiple treatments with doxorubicin reduce the papilloma frequency by only about 33% from week 10 onwards. Thus, in comparison to papillomas derived by a single initiating dose of DMBA which responded to multiple doxorubicin treatment by a reduction of 75%, papillomas induced by repeated DMBA applications are relatively more resistant to doxorubicin. It is of interest that the growth rates for the control groups over weeks 1 to 7 for both the single DMBA and the multiple DMBA papillomas are very similar (Fig. 4). The regression coefficients for the growth rates are 9.90 and 7.46 for the single DMBA and multiple DMBA control papillomas, respectively. It is therefore unlikely that differences in growth rate are the major determinant in the resistance of the papillomas to doxorubicin.

Response of Virally Initiated Papillomas to Doxorubicin. The multistage model of carcinogenesis of the mouse skin offers the option of initiating with a retrovirus carrying an activated Ha-ras oncogene rather than with a chemical. Mice initiated with HaMSV were split into 3 groups of at least 15 mice/group and treated with doxorubicin as described for chemically initiated papillomas. As Fig. 5 shows, a single acute dose of 10 mg/kg doxorubicin is as efficient as multiple injections of doxorubicin at reducing the frequency of papillomas in comparison to the control group. Both doxorubicin treatments reduce the frequency of papillomas by about 80% as calculated from week 10 onward. Papillomas arising as a result of single and multiple applications of DMBA responded to a single acute dose of doxorubicin by reductions of around 12 and 33%, respectively. Virally initiated papillomas are therefore more sensitive to doxorubicin treatment. From Fig. 4 it can be seen that the growth rate of the control virally initiated papillomas is greater than for the chemically initiated papillomas. The regression coefficient for the growth rate of the viral papillomas over

Fig. 3. Response to doxorubicin of papillomas initiated by multiple (14) applications of 25 μg DMBA. The response to doxorubicin of total papillomas (A) or papillomas >2 mm in two dimensions (B) is shown over time in weeks. The average papilloma number per mouse is the average of 25 mice/group counted at the time shown. Definitions of symbols are the same as legend to Fig. 1. Standard error (bars) is shown for B only.
CHRC5 reacted with the C219 antibody (35). C219 antibodiesensitive AUXB1 parental cell line and a drug resistant variant sensitivity to doxorubicin.

Attaining a size of 2 mm the viral papillomas show a greater ated groups. Thus despite the higher proportion of papillomas treatment in comparison to 10 to 20% in the chemically initi greater than 2 mm in two dimensions at the start of doxorubicin weeks 1 to 4 is 17.7. From Fig. 4 it can also be seen that around 50% of the total papillomas on virally initiated mice were greater than 2 mm in two dimensions at the start of doxorubicin treatment in comparison to 10 to 20% in the chemically initiated groups. Thus despite the higher proportion of papillomas attaining a size of 2 mm the viral papillomas show a greater sensitivity to doxorubicin.

Expression of P-Glycoprotein in Papillomas. Fig. 6A shows a Western blot analysis of proteins extracted from the drug sensitive AUXB1 parental cell line and a drug resistant variant CHRC5 reacted with the C219 antibody (35). C219 antibody detects all three P-glycoprotein isoforms and reacts cross species (35). P-glycoprotein is detected as a M. 170,000 band in the multidrug resistant CHRC5 line but not in the parental AUXB1 line (22).

Proteins extracted from normal mouse epidermis, viral papillomas, multiple DMBA initiated papillomas, single DMBA initiated papillomas, and papillomas from single DMBA initiated mice which have been treated with four doses of 5 mg/kg doxorubicin were transferred to nitrocellulose by dot blot apparatus. The filter was then reacted with C219 antibody in a manner identical to that for the Western blot (Fig. 6B). Fig. 6B shows that many of the papillomas from mice which had never been exposed to doxorubicin have levels of P-glycoprotein approaching that of the multidrug resistant CHRC5 cell line (Fig. 6B, Lane 5, rows a to d; Lane 11, Rows a to d; Lane 12, Rows a to d; Lanes 1 and 2, Rows e to h). There is also no obvious relationship between the level of carcinogen used to initiate the papillomas or the treatment of the mice with four doses of 5 mg/kg doxorubicin and the level of P-glycoprotein expression observed in the papillomas (Fig. 6B, Lanes 3 to 12, Rows e to h).

DISCUSSION

The ultimate failure of many common cancers to respond to chemotherapeutic agents has led to intensive studies on drug resistance (36, 37). The response of human tumors to chemotherapy varies enormously among tumor types and among individuals with the same histological tumor (36, 37). In a tumor, subpopulations of cells which have a genotype capable of conferring a drug resistant phenotype may arise spontaneously (8). However, exposure to carcinogens known to cause genetic instability and mutations may increase the probability of a cell acquiring a genotype associated with drug resistance (8, 38). Thus, it is the etiology of the tumor prior to drug exposure which may be most informative in determining the probability of resistance developing and the mechanisms of drug resistance relevant to the in vivo situation.

We have used the in vivo model of carcinogenesis of the mouse skin to examine whether the response of tumors to a chemotherapeutic agent, doxorubicin, is affected by differences in tumor initiation. Once papillomas started to appear (usually 4 to 6 weeks after initiation), TPA treatment was stopped and the animals were left for 1 week before injections with doxorubicin were started. In this way any effects that TPA might have on modulating the response of the papillomas to doxorubicin are avoided and only TPA independent papillomas will develop (39). It is important to notice that in all our experiments (Figs. 1, 3, and 5) no spontaneous regression in total papilloma frequency is observed. Fig. 7 summarizes the results by presenting the residual number of papillomas >2 mm remaining at more than 10 weeks after doxorubicin treatment expressed as a percentage of the untreated animals. A single dose of 10 mg/kg doxorubicin (Fig. 7A) is relatively inefficient at reducing the frequency of papillomas induced after either single or multiple treatments with the polycyclic hydrocarbon DMBA. In comparison, papillomas initiated by Harvey murine sarcoma virus are sensitive to a single dose of doxorubicin and show a reduction in the frequency of papillomas by more than 80%. The response of papillomas to 4 weekly injections of 5 mg/kg doxorubicin (Fig. 7B) shows that the single DMBA and viral Ha-ras initiated papillomas clearly respond to doxorubicin while there is little response from papillomas derived from
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Fig. 6. Expression of P-glycoprotein in papillomas. A, Western blot analysis of P-glycoprotein expression with C219 antibody in the multidrug resistant cell line CHRC5 and its parental line AUXB1. Each lane was loaded with 50 µg protein. Lane 1, drug resistant CHRC5 cells; Lane 2, parental drug sensitive AUXB1 cells. Positions of the molecular weight markers are shown and are in thousands. B, dot blot analysis of P-glycoprotein expression in papillomas using C219 antibody. Serial dilutions of the proteins of 1:10 were applied to nitrocellulose by dot blot manifold starting at 50 µg protein. Lane 1, Rows a-d, drug resistant CHRC5 cells; Lane 2, Rows a-d, drug sensitive AUXB1 cells; Lane 3, Rows a-d, drug sensitive A2780 cells; Lane 4, Rows a-d, normal murine epidermal cells; Lanes 5, 6, and 7, Rows a-d, papillomas initiated with Harvey murine sarcoma virus; Lanes 8-11, Rows a-d, papillomas initiated by multiple applications of DMBA; Lanes 12, Rows a-d, and 1-4, Rows e-h, papillomas initiated by a single application of DMBA; Lanes 5-12, Rows e-h, papillomas from mice initiated by a single application of DMBA and treated with four doses of 5 mg/kg doxorubicin.

Fig. 7. Summary of data on the differential response of papillomas initiated by chemicals and virus to doxorubicin. The frequency of papillomas >2 mm in any group of animals treated with doxorubicin was expressed as a percentage of the frequency of papillomas in their respective untreated control groups. These calculations were determined for all data after week 10 and the average values plotted. A relates the response of papillomas initiated by single or repeat (Rpt, 14 applications) doses of DMBA and virus (v-H-ras) to a single dose of 10 mg/kg doxorubicin. B relates the response of the papillomas to four weekly injections of 5 mg/kg doxorubicin.

multiple DMBA treatments. These data support the concept that the response of tumors to a chemotherapeutic agent is dependent on their exposure to carcinogen, with papillomas induced with the highest dose of DMBA being most resistant. Although the virally initiated papillomas have a faster growth rate than either the single or multiple DMBA initiated tumors, there is little difference in the growth rates between the two DMBA initiated groups (Fig. 4). It is therefore unlikely that growth rate of the papillomas is the major determinant in their response to doxorubicin.

The sensitivity of papillomas to doxorubicin could be related to intrinsic differences in target cells for these initiators or due to different molecular changes induced by the initiators. Papillomas induced by any single initiation protocol are known to be a heterogeneous group with respect to polyclonality, regression probability, TPA dependence, progression to carcinomas, and aneuploidy (40-48). Alternative initiation procedures as used in this study may therefore lead to quite distinct populations of papillomas (49). During promotion with TPA it is most likely that initiated cells which are resistant to the differentiation effects of TPA exposure develop into papillomas (49). Whether repeated exposure (Figs. 1 and 3) to DMBA gives rise to a comparable group of papillomas is unclear. Our results demonstrate that fewer papillomas develop during multiple DMBA exposure. Whether these are subsets which exist in the single DMBA initiated group or are a distinct group derived from a completely different stem cell is unknown. If indeed the target cell for transformation is different for the three different initiation protocols used, the inherent sensitivity to doxorubicin of the target cell for transformation and its progeny may determine the survival of papillomas during drug treatment. However, histological examination of representative papillomas from each initiation group showed them to be similar squamous cell papillomas derived from normal stratum spinosum (data not shown). This histological uniformity between papillomas derived by different initiation protocols does not exclude molecular heterogeneity.

Papillomas are dynamic lesions which pass through a number of well defined stages in terms of initiation, conversion, promotion, and progression (5, 23). Papillomas at different stages of promotion display an increasing tendency toward aneuploidy which may be related to increasing malignancy (45-48). The genetic heterogeneity observed may be the underlying reason for the emergence of a drug resistant subpopulation of cells within a papilloma (8). It is well documented that drug resistant variants can arise in a population of tissue culture cells and that the frequency of this event can be increased by exposure to mutagenic agents (8, 10, 38, 50). It would seem reasonable that one interpretation of the results is that the initiation agent acts on a population of target cells to introduce a number of alterations into the genome. Increasing the exposure to carcinogen would increase the probability of the development of drug resistant subpopulations. Alternatively, carcinogen exposure might induce genes involved in the cells response to toxic insult. Adaptive responses leading to an increased resistance to drugs including doxorubicin have been suggested to take place in hepatocytes from rats which have been chronically exposed to the carcinogen 2-acetylaminofluorene (12). Burt and Thorgeirsson (18) have shown that the administration of various xenobiotics to rats and mice induces expression of cytochrome P-450α, and P-glycoprotein suggesting that these and other genes
involved in the protection of cells from toxic substances may be regulated on a coordinate manner. The administration of DMBA to the mouse skin may therefore switch on a battery of genes not all of which are necessary to metabolize the carcinogen but are part of an overall detoxification system. However, since the response of papillomas to doxorubicin is related to initiation but is observed at least six weeks after initiation, this phenotype must be stable and not due to a transient effect of the initiator.

The mechanisms by which cells can become resistant to anticancer drugs including doxorubicin have been extensively studied in cell lines selected for survival in increasing levels of drug (21, 22). Cell lines derived in this manner have been most informative about the molecular changes concurrent with resistance and possible methods for circumventing resistance (22). Resistance to doxorubicin appears to be multifactorial with contributions from altered DNA repair, glutathione transferase activity, cytochrome P-450 activities, and topoisomerase II activity (22, 26, 51, 52). However, one of the most consistent changes observed in cell lines selected for resistance to doxorubicin and other natural drug products is high levels of expression of the Mr 170,000 P-glycoprotein which is capable of affecting drug efflux (22). P-glycoprotein isoforms are encoded by three genes in mouse and hamster and two genes in humans (22, 35, 53). We have analyzed the levels of P-glycoprotein expression in papillomas using the C219 antibody (35). Fig. 6B shows that the levels of P-glycoprotein expressed in papillomas prior to doxorubicin exposure are relatively high and can approach levels detected in the multidrug resistant CHRC5 cell line. The amount of P-glycoprotein observed in the papillomas does not appear to be related to the dose of carcinogen applied or injection of the mice with doxorubicin. Not all isoforms of P-glycoprotein can cause resistance to doxorubicin (53). In mouse, two of the three isoforms will cause drug resistance (53). The C219 antibody recognizes all three isoforms of P-glycoprotein (35) and therefore gene specific antiserum or probes will have to be used to determine which member of the P-glycoprotein family is expressed in the papillomas (53). However, due to the relatively high levels of P-glycoprotein expression observed in all the papillomas it is unlikely that P-glycoprotein expression is the major factor underlying the pattern of papilloma survival observed in each of the initiation protocols.

Experiments designed to determine the sensitivity of initiated stem cells to doxorubicin are in progress to assess whether clonal expansion is required before the resistance phenotype is observed, as is further molecular analysis of papillomas which have survived doxorubicin treatment for the expression of genes implicated in drug resistance (22, 26, 51, 52). This model is also ideally suited to pharmacological studies on drug distribution between normal tissues and papillomas and the heterogeneity between papillomas. By such studies it is hoped that a better understanding of the in vivo development of drug resistance may be achieved. The tumors develop on the backs of animals; thus their response to anticancer agents can be monitored over a time period without sacrificing the animal. In this way quantitative results on tumor numbers and size are obtainable as are the responses of individual tumors. Papillomas are discrete growths which can be removed for molecular or pharmacological studies. It should therefore be possible to relate the response of tumors to drug-tumor interactions or the expression of specific genes. A further advantage is that the mouse is ideally suited for somatic (29) or germ line (54) introduction of genes suspected of modifying anticancer drug efficacy.

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