SHORT COMMUNICATION

Medroxyprogesterone acetate accelerates the development and increases the incidence of mouse mammary tumors induced by dimethylbenzanthracene

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Chemical induction of mammary tumors in mice requires usually a long latency period and is often complicated by high non-mammary tumor related mortality. Classically hormone stimulation has been used as the means to increase tumor incidence. The synthetic progestin medroxyprogesterone acetate (MPA) was postulated by some authors to increase mammary tumor incidence in various rodent models. However, controversy exists regarding the role of MPA in experimental and human carcinogenesis. In our study we tested the use of a protocol of combined MPA- and dimethylbenz[a]anthracene (DMBA) treatment for the obtention of mammary tumors with a short latency and with a lower toxicity than the classical multiple dose DMBA protocol. MPA was very effective in accelerating the development and increasing the incidence of mammary tumors induced by DMBA in CD2F1 mice. MPA by itself did not produce any mammary tumors. The mean latency for tumor development from the end of carcinogen treatment was 99 ± 51 days in the group that received a combination of MPA and four DMBA doses. This group showed significantly earlier mammary tumor incidence (P < 0.0001) and higher tumor numbers than the groups that received only DMBA. Mammary tumors were also analyzed for effects on the mutation rate affecting the Ha-ras and Ki-ras genes. Our data is consistent with MPA probably increasing the number of target cells at risk for mutation by the chemical carcinogen DMBA and possibly promoting the faster development of tumors.

Rodent models of chemically-induced mammary carcinogenesis have been used over the years in order to gain insight into the biology of breast cancer. However, marked differences exist between rats and mice in their susceptibility to mammary tumor development after chemical carcinogen treatment. It is known that in general, laboratory mice are much more resistant to chemical induction of mammary tumors when compared with rats. For instance in rats a single dose of 5–10 mg/100 g b.w. dimethylbenz[a]anthracene (DMBA*), without any exogenous hormonal manipulation, is sufficient to induce a high incidence of mammary tumors (1); while mice require a regimen of multiple applications of comparable doses of DMBA to obtain a relatively modest tumor response with a long tumor latency, high morbidity and mortality non-mammary tumor related (2–4). Rat mammary tumors are largely hormone dependent for growth, while most mouse mammary tumors once developed become hormone independent. Partially, for these reasons the rat mammary tumor models are much more widely used than the mice models. In our laboratory we have been using rat mammary tumor models to elucidate the role of critical genetic and chromosomal events in mammary carcinogenesis (5,6). However an obvious problem is the relative paucity of information at the genetic level in the rat (7). Because of more extensive knowledge of the mouse genome, it would be expedient to use mouse models for comparison with findings in human breast cancer.

One of the goals of the studies reported here, was to develop a simple and reliable protocol for the chemical induction of mouse mammary tumors with relatively short latency and high tumor incidence. It is very well documented that hormonal stimulation is a requisite for obtaining a high mammary tumor incidence in the mouse (2–4,8). Exogenous hormone administration and pituitary isografts have been classically used (8,9). Recently, Pazos et al. reported that multiple N-methyl-N-nitrosourea (NMU) doses in combination with medroxyprogesterone acetate treatment (MPA) were very effective in the induction of mouse mammary adenocarcinomas in BALB/c mice (10). Based in part on these observations and to clarify some of the controversy on the role of MPA in carcinogenesis, in our study we tested the use of a protocol of combined MPA–DMBA treatment for the obtention of mammary tumors with a short latency and with a lower toxicity than the classical multiple dose DMBA protocol. In order to obtain some information on the molecular mechanisms of carcinogenesis, tumors induced with this protocol were also analyzed for the incidence of mutations in Ha-ras and Ki-ras genes.

Virgin female CD2F1 (Balb/C×DBA/2) mice purchased from the National Cancer Institute (Fredericksburg, MD) were randomized and separated into four groups, Groups 1–4 (housed 10/cage) of ~40 mice each for the different treatment protocols (Figure 1) and one control group 5 (MPA only) of 20 mice. At 6 weeks of age, animals from Groups 3, 4 and 5 received a subcutaneous implant in the interscapular area of two compressed pellets, standard release no binder, of medroxyprogesterone acetate, 20 mg each (Hormone Pellet Press, Kansas City, KS). DMBA (Sigma) at a concentration of 1 mg/dose, was administered intragastrically dissolved in 0.1 ml cottonseed oil at the time points indicated in Figure 1. Animals were monitored weekly by palpation for mammary tumor development.

Mice were euthanized when palpable mammary tumors reached ~1.5 cm in diameter. A part of tumor tissue was frozen for DNA analysis and a part was fixed in formalin, embedded in paraffin and processed for standard hematoxylin–eosin staining. Tumor histologies were classified as previously described (2).

For the statistical analysis of tumor incidence curves and tumor latency, time 0 was considered as the time when
Fig. 1. Mammary carcinogenesis treatment groups in CD2F1 mice.

Fig. 2. Cumulative proportion of mice without mammary tumors, using Kaplan-Meier survival statistics. Treatment groups as indicated in Figure 1. Group 4 showed a significantly earlier (P < 0.0001) mammary tumor incidence than groups 1, 2 and 3 Group 5, with zero tumor incidence was not plotted.

The time to tumorigenesis was noted for each animal. Causes of morbidity and mortality other than mammary tumors were considered as censored observations. The time to tumorigenesis was analyzed using Kaplan-Meier survival statistics with Gehan-Breslow significance test (Statistica: CSS, Tulsa, OK). Tumor latencies, unadjusted for censoring, were also compared by using the Student’s t-test. Animals were observed for a total of 15 months.

Ten randomly selected tumors from group 4 and ten from the DMBA only groups (1 and 2) were analyzed for mutations in exons 12/13 and 61 of the Ha-ras and Ki-ras genes. Genomic DNA (0.2 μg) was added to a 10 μl reaction containing 0.5 mM of each dNTP, 6 pmol of each primer, 0.6 U of Taq polymerase and 5 μCi (a-32P)dCTP in amplification buffer (50 mM KCl, 10 mM Tris, pH 8.3, 1.5 mM MgCl2). Ha-ras amplifications were performed using primers previously reported (11). Primers used for Ha-ras exon 1 amplification included 5'-GCTAAGTGTCCTCTCATGGG-3' and 5'-CTCTATAGTGGAATCTACTACG-3'. Primers for Ha-ras exon 2, included 5'-GACTCTACCGGAAACAGGT-3' and 5'-TGATGGATGTCCTCGAAGGA-3'. Primers for amplification of exons 1 and 2 of Ki-ras were selected based on published sequence (12). They included 5'-GTAAGGCCTGCTGAAATAG-3' and 5'-GGTGTCACTCATCCACAA-3' (Ki-ras exon 1); 5'-GACTCTACGGAAACAGGT-3' and 5'-GGTGAATATCTTCAAATGAT-3' (Ki-ras exon 2). After an initial denaturation step at 95°C for 2 min, 40 cycles were performed at 95°C for 30 s and either 51°C (Ha-ras exon 1 and Ki-ras exon 2), 55°C (Ki-ras exon 1) or 60°C (Ha-ras exon 2) for 30 s, and 72°C for 1 min for all four exons.

Single strand conformation polymorphism (SSCP) gels were run in order to screen potential point mutations (13). Two different gel systems were used to screen for mutations. In gel system A, gels containing 6% polyacrylamide (acrylamide:bis, 19:1), 10% glycerol and 1xTBE (1xTBE = 90 mM Tris-borate, 2 mM EDTA) were electrophoresed in 0.5xTBE at 4 W for 14 h at room temperature and were used to resolve [a-32P]dCTP-labeled PCR products from exon 1 (including codons 12/13) of both the Ha-ras and Ki-ras genes. In gel system B, gels contained 0.5xMDE acrylamide (AT Biochem) and 0.6xTBE and were electrophoresed in 0.6xTBE at 4 W for 14 h at room temperature. They were used to resolve mutations in exon 2 (including codon 61). Aliquots of the PCR reactions were diluted 1:5 in 95% formamide, 10 mM NaOH, 0.05% bromophenol blue and 0.05% xylene cyanole. Samples were heated at 95°C for 5 min and cooled on ice. Typically, 5 μl of this mixture were electrophoresed. Gels
humans as a contraceptive, in the treatment of breast cancer, induced mammary tumors. The development and increased the incidence of the DMBA protocol was Type B adenocarcinomas (2), i.e. with no squamous metaplasia. Table II. Interestingly however, a high proportion of the tumors generated in Group 3, (MPA-DMBA X 2) were of the tubular type (Type A). The most important conclusion from our study is that MPA accelerated the growth of the mammary neoplasias induced by DMBA. Nevertheless, we cannot rule out that MPA could be acting as a co-

were dried on blotting paper, and exposed to X-ray film. Gel bands from samples that were shifted relative to those from wild type DNA were excised from the dried gel, eluted reamplified and sequenced as previously described (14).

In Group 1 of the tumor experiment (Figure 1), we followed the conventional multiple DMBA dose treatment (2,4), six 1 mg doses per mouse. We observed that this protocol was extremely inefficient in the generation of mammary tumors, see Table I. This was due to the very high morbidity and mortality during the course of the experiment for causes other than mammary tumors. In Group 2, where DMBA was given four times, morbidity and mortality were much lower. As a result, mammary tumor incidence was higher, although considerably less than the tumor incidence obtained in the MPA–DMBA treated groups. The combination of MPA with four DMBA doses (Group 4) was the most efficient protocol inducing tumors with the shortest latency and lowest non-mammary tumor mortality (Figure 1) in CD2F1 mice.

Group 4 showed a significantly earlier mammary tumor incidence curve (P < 0.0001) than Groups 1, 2 and 3 (Figure 2). The first tumor arose at 42 days, counted from the time that all treatments ended at week 13. Fifty percent of the mice had tumors by ~18 weeks from that time point. This protocol also showed, the highest tumor incidence since 21 of 30 (70%) mice developed mammary tumors, and tumor multiplicity was also the highest with 1.17 mammary tumors/mice (Table I). Almost all of the mammary tumors induced by the MPA–DMBA protocols were Type B adenocarcinomas (2), i.e. with no squamous metaplasia, Table II. Interestingly however, a high proportion of the tumors generated in Group 3, (MPA–DMBA X 2) were of the tubular type (Type A). The most important conclusion from our study is that MPA accelerated the development and increased the incidence of the DMBA induced mammary tumors.

Depot medroxyprogesterone acetate is widely used in contraception, in the treatment of breast cancer, endometriosis, and other pathological conditions (15). Controversy surrounds its use and potential influence on the risk for developing breast cancer (16). Recently, it was concluded that there is an increase in developing breast cancer only in recent or current MPA users, with the highest risk seen when used 5 years or less prior to the development of breast neoplasia (17). These authors further speculated that one of the possible explanations would be that depot MPA may accelerate the development of preexisting tumors (17).

The results of our studies are analogous to previous observations of Pazos et al. (10) who demonstrated that MPA in combination with the direct alkylating agent nitrosourea was effective in the induction of mammary tumors in Balb/C mice, in our experiment, however, we used a much lower dose of MPA. Several years ago Lanari and coworkers (18) reported that MPA depot alone at very high doses, 40 mg every 2 months during a year, was sufficient to induce mammary tumors in Balb/C mice after a very long latency period. In our experiment, however, MPA by itself at the dose of 40 mg MPA once in s.c. pellet implants, did not induce any mammary tumors during the observation period of 15 months. This indicates that in this system, MPA fits the operational definition of a tumor promoter, since it was not, by itself, carcinogenic but enhanced the tumor response and promoted the tumor growth of the mammary neoplasias induced by DMBA. Nevertheless, we cannot rule out that MPA could be acting as a co-carcinogenic agent and that the dose utilized in our study is sub-threshold for the generation of tumors by itself but still sufficient to observe a synergistic effect with other carcinogens. Russo and Russo (15) have shown that MPA at a single 5 mg dose, equivalent to the human pharmacologic dose, was sufficient to induce a very significant cell proliferative response in the rat mammary terminal end buds, inhibiting differentiation. These authors showed that pretreatment with MPA also increases the tumor response in the DMBA rat model (15,19). On the other hand, other investigators have reported that

Table I. Influence of MPA treatment on formation of DMBA induced mammary tumors in CD2F1 mice

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of mice</th>
<th>Total mammary tumors (MT)</th>
<th>Mice with MT</th>
<th>MT/mouse</th>
<th>Latency for MT development (days) X (± SD)</th>
<th>Non-MT related deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (DMBA×6)</td>
<td>32</td>
<td>8</td>
<td>5/32 (16%)</td>
<td>0.25</td>
<td>152 ± 75</td>
<td>26 (81%)</td>
</tr>
<tr>
<td>2 (DMBA×4)</td>
<td>35</td>
<td>24</td>
<td>15/35 (43%)</td>
<td>0.69</td>
<td>218 ± 72</td>
<td>18 (51%)</td>
</tr>
<tr>
<td>3 (MPA-DMBA×2)</td>
<td>36</td>
<td>28</td>
<td>21/36 (58%)</td>
<td>0.78</td>
<td>210 ± 65</td>
<td>11 (31%)</td>
</tr>
<tr>
<td>4 (MPA-DMBA×4)</td>
<td>30</td>
<td>35</td>
<td>21/30 (70%)</td>
<td>1.17</td>
<td>99 ± 51&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8 (27%)</td>
</tr>
<tr>
<td>5 MPA</td>
<td>20</td>
<td>0</td>
<td>0/20 (0%)</td>
<td>0.0</td>
<td>–</td>
<td>0.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Latency for tumor development from the end of all treatments at week 13 (time 0) unadjusted for censoring.<br><sup>b</sup>Statistical significance versus Group 4 = /<br><sup>c</sup>Includes deaths due to other neoplasias. these were: Group 1 = 3 mice, Group 2 = 4 mice, Group 3=4 mice and Group 4 = 5 mice.

Table II. Mammary tumors histopathological classification

<table>
<thead>
<tr>
<th>Group</th>
<th>Total tumors</th>
<th>Type B adenocarcinomas&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Type A adenocarcinomas</th>
<th>Adenosquamous carcinomas&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Myoepithelial adenocarcinomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>5 (63%)</td>
<td>1 (12%)</td>
<td>1 (12%)</td>
<td>1 (12%)</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>15 (63%)</td>
<td>5 (21%)</td>
<td>3 (13%)</td>
<td>1 (4%)</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>16 (57%)</td>
<td>8 (29%)</td>
<td>3 (11%)</td>
<td>1 (4%)</td>
</tr>
<tr>
<td>4</td>
<td>35</td>
<td>25 (71%)</td>
<td>2 (6%)</td>
<td>6 (17%)</td>
<td>2 (6%)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Includes well and poorly differentiated carcinomas.<br><sup>b</sup>Includes tumors with >10% of focal squamous metaplasia.
pretreatment with MPA inhibits the DMBA tumor response in rats (20). Controversies such as this one, using the same experimental system (19,20), exemplifies the need for further studies to better define and clarify the role of MPA in mammary carcinogenesis.

In order to analyze the level of involvement of some known putative molecular mechanisms of chemical carcinogenesis we also analyzed the occurrence of mutations in the Ha-ras and Ki-ras proto-oncogenes in the mammary adenocarcinomas induced by DMBA alone and compared these with mutations found in those tumors induced by MPA–DMBA. It was of interest for us to explore whether the MPA treatment affected the rate and/or type of mutations affecting this oncogene family. Ha-ras codon 61st mutations have been reported previously in DMBA induced mouse mammary tumors. However, in those studies tumors were generated from hyperplastic (preneoplastic) mammary outgrowth lines (21,22), no information is available on the true frequency of Ha-ras mutations in ‘primary’ DMBA induced mouse mammary tumors. Mutations affecting the Ki-ras gene (codon 12) have been reported in NMU induced mouse mammary tumors (9). In our analysis we found that three tumors out of ten analyzed (30%) from the MPA–DMBA group showed activating mutations in codon 61st of the Ha-ras gene versus only one tumor out of ten (10%) analyzed in the DMBA group had a mutation. On sequencing these, all four samples showed a CAA→CTA transversion (Gln→Leu) in codon 61 (Figure 3). No other mutations were observed in either codons 12 or 13 of Ha-ras or in the Ki-ras gene codons 12, 13 and 61. We can conclude that MPA pretreatment did not generate any novel mutations in Ha-ras or Ki-ras, however it appeared to increase the rate of Ha-ras codon 61st mutations generated by DMBA.

Although our original objective was to develop an efficient mouse mammary chemical carcinogenesis protocol, it is relevant that our findings add weight to previous reports that postulated that MPA can promote the development of mammary tumors both in rats and mice (10,19). As suggested by Russo and Russo (15), it is possible that MPA could act by generating a proliferative response in a specific compartment of the mammary gland, thus increasing the number of target cells at risk for mutation by the carcinogen treatment. Still much has to be learned to fully understand the effect of this potential tumor promoter on the mammary gland.

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References


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