Skin Nucleic Acid Phosphorus Metabolism of DBA/1J Mice during Implanted Tumor Development and Methylcholanthrene Carcinogenesis

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SUMMARY

Implanted tumors and methylcholanthrene carcinogenesis increase the turnover of phosphorus in the total nucleic acids of histologically normal DBA/1J mouse skin. As the implanted tumors progress for 6 days, the turnover rate of nucleic acid phosphorus in the histologically normal skin increases to 4 times that of control skin from normal DBA/1J mice. At this same time, 68% of the histologically normal skin implants from the tumor-bearing mice develop into lymphosarcomas when implanted into normal DBA/1J mice.

Painting mice on the dorsum with methylcholanthrene results in a decrease in the turnover rate of nucleic acid phosphorus of the painted skin. However, the nucleic acid phosphorus turnover of ventral unpainted skin from the same mice is increased as painting progresses. After six weeks of painting, the ventral unpainted skin, though histologically normal, develops into a lymphosarcoma when implanted into normal DBA/1J mice. This transplantability occurs after the appearance of papillomas on the painted area.

INTRODUCTION

In a previous publication (6), using a strain of mice (C57BL/6) resistant to methylcholanthrene carcinogenesis, we reported that, under in vitro conditions, the specific activity of nucleic acid phosphorus was higher in histologically normal epidermis from tumor-bearing mice than in histologically normal epidermis from normal mice of the same age. A similar pattern of incorporation of radioactive phosphorus was also found in vivo. However, the differences observed in vivo were not as great as the differences found in vitro.

It became of interest to determine whether similar malignancy associated changes could be demonstrated under in vivo conditions in the skin of DBA/1J mice which are susceptible to carcinogenesis as (a) tumors develop after implantation and (b) as chemical carcinogenesis takes place.

This report presents a study using a strain of mice susceptible to methylcholanthrene carcinogenesis (DBA/1J). It compares the nucleic acid phosphorus metabolic activity of adjacent histologically normal skin as tumors develop after implantation and as methylcholanthrene carcinogenesis takes place with NA-P activity in normal skin from normal animals.

MATERIALS AND METHODS

Implant Studies

DBA/1J mice, four to six weeks old, obtained from Jackson Memorial Laboratory, Bar Harbor, Maine, were implanted with a tumor (lymphosarcoma) which is being studied in our laboratory. This tumor line was produced by implanting into the isologous strain a lymphoid tumor from a DBA/1J mouse which had been painted as described below. The tumor is in its 54th passage and kills the mice in an average of 12.5 days. The implanted mice were sacrificed at 2-day intervals for chemical studies. Unimplanted mice of the same strain and age at sacrifice served as normal controls.

Painting Studies

DBA/1J mice of the same age and source as described above were painted on the dorsal unepilated interscapular skin with a solution containing 0.6% 20-methylcholanthrene in reagent grade benzene using a No. 4 camel's hair brush. Painting was carried out five times a week for nine weeks. Epilation occurred after one week's painting; papillomas appeared by the sixth week; and squamous cell carcinomas appeared during the ninth week after the onset of painting and thereafter. The animals were sacrificed at two-week intervals for chemical studies. Untreated mice of the same strain and age at sacrifice served as normal controls.

Chemical Analyses

The following procedure for chemical analyses was identical for the implanted, the painted, and the normal mice. Twelve hours prior to sacrifice the mice were inoculated subcuta-
ously with 3 μc of $^{32}\text{P} (\text{NaHPO}_4)$ per gram of body weight. Time-incorporation studies (performed in our laboratory) demonstrated that a twelve-hour incorporation of phosphorus is on the ascending part of the time-incorporation curve of phosphorus into skin nucleic acids.

The mice were sacrificed by cervical dislocation and immediately chilled in an ice bath; subsequent procedures were carried out at approximately 5°C. The hair was removed by shaving, and the subcutaneous tissue was dissected away. Skin removed during both the implant and painting studies was taken from a site on a side opposite from the tumor or opposite from where the painting was done. The following extraction procedure is a modification of the Schmidt-Thannhauser method (7) as modified by Huggins and Cohn (4). In each case approximately 150 mg of skin were homogenized and extracted with 5 ml 10% cold TCA. The residue was washed 2 times with 10% cold TCA, 2 times with cold distilled water, and 2 times with cold acetone. The phospholipids were removed by extracting 3 times with chloroform:methanol (2:1) at 60°C. The total nucleic acids were extracted 3 times with 10% NaCl in a 100°C boiling water bath and precipitated with 2 volumes 95% ethanol at −10°C. The precipitated nucleic acids were dissolved in 5 ml of deionized water. The nucleic acid fraction was tested by the procedure of Lowry et al. (5), and no detectible protein was found.

The total phosphorus content of the nucleic acids was determined by the method of Bartlett (1). The activity of phosphorus was assayed using a Geiger-Mueller detector. Counts per minute were corrected for background and to Day zero (day of inoculation with $^{32}\text{P}$).

Specific activity was calculated as cpm per μg of phosphorus per 100 mg of tissue. The percent RSA [a measure of the complete turnover rate (2)] was calculated as the specific activity of the nucleic acid fraction divided by the specific activity of the TCA-soluble fraction times 100. This calculation was made to eliminate variations in the activity of the radioactive TCA-phosphorus pool which occurred from mouse to mouse. This TCA-phosphorus pool is the source of phosphorus incorporated into the nucleic acids.

For pathologic examination, skin was fixed in formalin, dehydrated in a series of graded alcohol concentrations, embedded in paraffin, and stained with hematoxylin and eosin.

**Criterion for Malignancy**

The ability of the skin to produce tumors was assayed by implanting a small piece of the skin subcutaneously into the axillary region of a mouse of the same strain, using a 12-gauge trocar needle. The recipient animals were observed daily for tumor progression. The tumors that develop from the implant of histologically normal skin progress with the same rapidity as do tumor implants.

**RESULTS**

**Implant Studies**

Chart 1 compares the NA-P turnover rate [RSA] (2) of histologically normal skin from the tumor-bearing mice at various time intervals during tumor development with that of normal skin from unimplanted controls of the same age. The mean value 6.8 ± 2.6 for normal skin is a mean of 10 determinations from 10 normal mice that were the same age as the treated mice. Each of the other points is the mean of 6 determinations from 6 mice. The vertical bar at each point on the time curves and at each point on the normal control value is ± the standard deviation. Two days following implant, the NA-P RSA doubled and by 6 days after implant it was 4 times that of normal. After reaching a peak 6 days postimplant, the activity fell to normal at 10 days. The NA-P content remained relatively stable throughout the first 8 days of the study period.

Table 1 shows that five days postimplant, the histologically normal skin from the tumor-bearing mice, when implanted into mice of the same strain, developed into a lymphosarcoma in 30% of the recipients. Six days after implant, 68% of the normal-appearing skin implants developed into lymphosarcomas when implanted into the same strain of mice. Implanting mice with normal tissues from control animals produced no alteration in NA-P metabolism similar to that observed during tumor progression.

<table>
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<tr>
<th>Duration of tumor on mouse from which skin implant was made (days)</th>
<th>Number of animals developing tumors from skin implant</th>
<th>Percent of animals developing tumors from skin implant</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0/20</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0/20</td>
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<td>68</td>
</tr>
<tr>
<td>8</td>
<td>44/50</td>
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Incidence of lymphosarcomas resulting from implant of histologically normal skin from mice receiving lymphosarcoma implants.

**RESULTS**
Painting Studies

Chart 2 compares the turnover rate (RSA) of NA-P of the methylcholanthrene-painted skin with that of normal skin from normal, untreated animals over a period of ten weeks. After two weeks of painting, the turnover of NA-P of treated skin increased to 4 times that of normal skin. This activity began to decrease as painting continued. A 50% decrease in activity was observed one week after the painting was discontinued.

The effects of dorsal methylcholanthrene painting on the NA-P turnover of histologically normal skin from the unpainted ventral surface of mice are seen in Chart 3. At 2 weeks, the RSA was over 2 times that of normal skin, and, after painting was stopped at 9 weeks, it continued to increase to over 20 times that of normal skin at four to six weeks after tumor development. Only after the development of papillomas did this histologically normal skin, showing the increased NA-P metabolism, develop into a lymphosarcoma when implanted into normal mice of the same strain. Table 2 shows that, after 6 weeks of painting, 15% of the histologically normal skin implants from the methylcholanthrene-treated mice developed into lymphosarcomas in the recipients. After nine weeks of painting, 40% of the implants of histologically normal skin developed into lymphosarcomas. Thirty-nine weeks after the last painting, 70% of the histologically normal implants developed into lymphosarcomas.

It should be pointed out again that the skin used in the chemical and implant studies was taken from a site on the animal as far away from the painting or developing tumor as possible.

Painting the mice with benzene only, the vehicle for the methylcholanthrene, produced no alteration in NA-P metabolism or implantability. These results were similar to those observed during painting with methylcholanthrene.

DISCUSSION

Hardin et al. (3) reported the ability of methylcholanthrene to stimulate squamous cell carcinomas on the skin of DBA/1J mice. They also showed that the transfer of normal-appearing ventral skin from tumor-bearing mice to untreated DBA/1J mice resulted in lymphosarcoma. Scholes et al. (6) later demonstrated, in C57BL/6 mice, an increased metabolic activity in histologically normal epidermis obtained from mice bearing a squamous cell carcinoma induced with 20-methylcholanthrene. This tissue had a phosphorus turnover more closely resembling neoplastic rather than normal tissue.

The NA-P turnover of skin of mice implanted with a lymphosarcoma reaches a peak in activity midway between the time of implant and the average time postimplant that the animals die. It is noteworthy that just prior to this peak in activity the implanting of this histologically normal skin into untreated DBA/1J mice causes the implants to develop into

<table>
<thead>
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<th>Weeks following</th>
<th>Number of animals</th>
<th>Percent of animals</th>
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</thead>
<tbody>
<tr>
<td>initiation of painting</td>
<td>developing tumors from skin implant</td>
<td>developing tumors from skin implant</td>
</tr>
<tr>
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<td>0/10</td>
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<td>48</td>
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Incidence of lymphosarcomas resulting from implant of histologically normal ventral skin from mice painted dorsally with 20-methylcholanthrene.
lymphosarcomas. We have homogenized this tissue, filtered it through a 0.2μ filter, and inoculated the animals with a filtrate of the same strain. No tumors of any kind have resulted from passage of this filtrate. In addition, electron microscope studies of glutaraldehyde-fixed and Epon-embedded tissue show no evidence of virus-like particles in this transplantable tissue.

As can be seen from this study, the NA-P turnover in painted skin increases rapidly during the first two weeks of painting then decreases as painting continues. However, the NA-P turnover in adjacent, nonpainted, histologically normal skin increases gradually during painting, and, after papillomas develop, it increases to higher values as the malignant process develops. NA-P turnover remains high until the malignant process causes a severe debilitation of the animal. Only after the appearance of papillomas does this transfer of histologically normal ventral skin to untreated DBA/1J mice result in a lymphosarcoma. Heterologous transfer of this tissue into different mouse strains results in rejection.

The literature abounds with reports regarding malignancy associated changes. However, many of these reports the use of animals which carried tumors for many weeks or months and were therefore in the terminal stage of the disease. The work presented here points out the importance of determining a time-activity relationship of tumor development when studying malignancy associated changes. The correlation between day of implant or extent of chemical carcinogenesis, NA-P activity, and the lymphosarcoma-producing capacity of histologically normal skin points this out.

Many of the reports in the literature compare the chemistry of tumor tissue with normal tissue of the same animal. This study demonstrates a chemical change in the NA-P metabolism of tissues of the tumor-bearing host which are not apparently infiltrated with the malignant process and appear histologically normal. Therefore, normal adjacent tissue of the tumor-bearing host should not be used as a normal control in such comparative studies.

REFERENCES

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