Stage-dependent Induction of Prenatal Tumors in Mice by the Kirsten and Moloney Strains of Murine Sarcoma Viruses

Lorenzo Rossi, Ottavia Barbieri, Simonetta Astigiano, Donatella Ugolini, and Oliviero Varrici

ABSTRACT

The Moloney (MoMSV) and Kirsten (KiMSV) strains of murine sarcoma viruses are known to induce mesenchymal sarcomas upon infection of newborn rodents. To determine their activity in mouse embryos, 11- to 15-day-pregnant CD-1 mice were laparotomized, and the single implants were inoculated into the abdominal portion of the embryonal body with an average of 15 and 1500 focus-forming particles/g of body weight of the MoMSV and KiMSV viruses, respectively. Another group of less than 1-day-old pups was given a comparable amount of either virus. Tumors appeared in the young within the first few weeks of life with incidences and histological types dependent on the gestational day and the viral strain inoculated. Mixed mesenchymal sarcomas at or near the site of inoculation and vascular tumors of the brain were by far the most frequent neoplasms observed in the newborn. With MoMSV there was an increased incidence of sarcomas with advancing age at treatment, being 0% at 11 days of pregnancy and 96% in newborn (P for trend, <0.025). By contrast, KiMSV caused an incidence of sarcomas below 20% throughout (P for trend, >0.05).

Brain tumors were identified in the several MoMSV and KiMSV groups, with a peak value of 43% following the inoculation of both viruses into 13- and 15-day-old embryos, respectively. While the total incidence of these tumors was significantly different from controls, no positive trend by day of treatment was found among the MoMSV and KiMSV viruses (P < 0.05). The tumors were mainly capillary angiomas, but a few cavernous angiomas were also detected. In addition, eight pups which were given injections of both viruses at developmental Days 11 to 13 had tumors of the choroid plexus. In many instances, newborn pups were affected by multiple vascular abnormalities of the brain, including capillary telangiectases and multiple hemorrhagic areas. No such lesions nor tumors at any site were found among the control animals.

The present results are important not only because of the evidence that Swiss embryos respond selectively to the carcinogenic effects of murine sarcoma viruses, but also because they offer the opportunity to dissect directly in vivo the mechanisms underlying the stage-related sensitivity of prenatal mice to oncogenic retroviruses.

INTRODUCTION

Studies on experimental transplacental carcinogenesis have repeatedly indicated that growing embryos can be a primary target of exogenous neoplastic stimuli derived from maternal exposure to environmental carcinogens (1, 2). At least in rodents, the vulnerability of prenatal animals to tumor induction is a stage-specific event, and it usually takes place not before the placenta has reached full maturation (3–5). Our knowledge of the various aspects of the reaction of embryonal tissues to carcinogens comes mainly from work with chemicals, particularly those which are genotoxic to immature cells (6, 7). Although prenatal neoplasms by other agents are much less documented, there exist a few reports dealing with the appearance of tumors in neonatal rodents infected with DNA and RNA tumor viruses at certain critical stages of postimplantation development (8–12). One of these studies, relevant to the present approach, concerns the production of mesenchymal sarcomas and other early life tumors following the direct injection of the MoMSV virus into rat fetuses (10). While, however, the carcinogenicity of this and other mammalian sarcoma viruses has been proved in newborn animals of various species (13), no data are yet available on their interaction with mouse embryos.

By using the intraembryonal injection technique as a means of gaining direct access to the gravid uterus, thus avoiding maternal and placental interferences, we are now investigating the carcinogenicity of selected murine retroviruses in pregnant rodents in terms of host and stage sensitivity and morphological appearance of tumors in the newborn. Here we show that the Moloney and Kirsten strains of the acutely tumorigenic MSV induce different incidences and multiple tumor types in Swiss mouse pups given injections during the second half of pregnancy. Since the cellular homologies of the transforming genes carried by KiMSV and MoMSV are expressed at different dose levels in mouse embryos (14–17), we compared the effects of both these viruses in the same model system, hoping the results would yield a range of information useful to dissect tumor induction and maintenance in prenatal animals. Altogether, our findings support the hypothesis that multiple mechanisms are involved in embryonal carcinogenesis by RNA tumor viruses.

MATERIALS AND METHODS

Viruses. The MoMSV virus was originally obtained from Dr. J. A. Levy, San Francisco, CA, and the KiMSV virus was from Dr. S. Rasheed, Los Angeles, CA. Both viruses were propagated at the Institute of Microbiology of the University of Genoa. Viral infectivity was determined by the focus formation assay (18). Briefly, viral dilutions were inoculated onto mouse NIH3T3 cells plated 24 h previously and pretreated with DEAE-dextran (25 µg/ml). After adsorption for 30 min at 37°C, the cells were refed and maintained in Dulbecco's modified minimal essential medium (Flow, Italy) containing 5% heated (56°C, 30 min) calf serum, 1000 units of penicillin/ml, and 1 mg of streptomycin/ml.

The abbreviations used are: MoMSV, Moloney strain of murine sarcoma virus; KiMSV, Kirsten strain of murine sarcoma virus; FFU, focus-forming unit(s); MSV, murine sarcoma virus.
glutamine (2 mM), and gentamicin (10 ml/liter) (19). Viral preparations of the KiMSV and MoMSV contained 1 x 10^{6.08} and 1 x 10^{3.39} FFU/ml, respectively.

Animals. Eight- to 10-wk-old male and female CD-1 mice (Charles River, Como, Italy) were mated overnight, and the morning a vaginal plug was found represented Day 0 of pregnancy. Pregnant and nursing mothers were fed a commercial diet (RF 25) especially balanced for this purpose; the weaned progeny received a standard diet (both from Italiana Mangimi, Milan, Italy). Throughout the experiment, food and water were supplied ad libitum.

Experimental Approach. The experimental groups, the number of embryos given injections (the average number of living implants was estimated of 10 embryos/female), and treatments are reported in Table 1. Fertilized mice were laparotomized on Days 11 to 15 of gestation, and all embryos found in the uterus were inoculated with the undiluted preparations of MoMSV and KiMSV; the volumes injected were 1, 1.25, 3, 5, and 10 µl/embryo on Days 11 to 15, respectively. These doses ensured that the animals received an average of 15 FFU of MoMSV per g of body weight and 1500 FFU of KiMSV per g of body weight at any developmental stage exposed. By using a microinjection apparatus consisting of a microneedle attached to a precision syringe, a micromanipulator, and a dissection microscope when needed, the viruses were injected directly into the embryonal body, carefully avoiding any contact with the head and any leakage of liquid that might inadvertently spread to maternal tissues. After treatment, the peritoneum and abdominal skin were su tutred separately with absorbable surgical suture (Dexon Cy-anamid, United Kingdom), and females were then placed in single cages and allowed to spontaneously deliver the pups. The control groups received the virus-free medium on Days 11, 12, or 14 of pregnancy. For consistency of results, litters with less than 50% of the injected embryos at delivery were discharged from the experiment. The living and dead young were checked for gross malformations and superficial nodules, left with their litter mothers, and weighed once a week until weaning. After weaning, the animals were distributed according to sex and treatment and examined every other day for signs of external or internal tumor development. Mice found dead or sacrificed because moribund and those surviving at 20 wk of age were necropsied, and abnormal organs and tissues were stained with hematoxylin-eosin for histological analysis. At least 4 cuts were performed in single brains. Selected sections of tumors were also stained with phosphotungstic acid-hematoxylin and with the Welsch and Del Rio Hortega methods. Pups which were cannibalized or excessively decomposed were excluded from the final evaluation. For this reason, the number of animals at risk in some experimental groups was reduced up to 50% of the original number counted at delivery.

Overall mortality and incidence of tumors of various types were evaluated by means of the methods of Mantel (20). (a) Mortality and tumor incidence in the controls were compared with those observed in the groups treated with either MoMSV or KiMSV by pulling together the groups treated at different days of development. (b) The presence of an association between tumor incidence and day of treatment was assessed by means of a test for trend.

RESULTS

Viability Rate. There was a high yield of viable pups (incidence ≥90%) following the injection of the virus-free medium into 11-, 12-, or 14-day-old embryos. On the contrary, the injection of MoMSV and KiMSV at 11 and 12 days of pregnancy caused a poor survival of the conceptuses of which between 55 to 85% were reabsorbed or stillborn, while, on the average, the embroy-ethal effects that resulted were low or negligible when later gestational stages were inoculated (Table 1). This different outcome might be explained on the basis that both viruses may have interfered with critical morphogenetic processes taking place at midgestation and, for example, we found that several pups born from 11- and 12-day-treated embryos were malformed and died within 24 h of life.

During the first few days after birth, the mortality rate prevailed among the groups treated prenatally with KiMSV compared to MoMSV and control groups. Thereafter, the tendency reversed, and the animals of the MoMSV groups started to die at a frequency in excess to that observed in the KiMSV groups (Table 1). The viruses had no influence on the body growth of neonates that gained weight regularly during the entire lactation period.

Pathological Findings. The MoMSV and KiMSV viruses but not the virus-free medium caused a variety of tumors in pre- and neonatally inoculated animals (Table 2). Interestingly more than 80% of these tumors were detected within the first 5 wk of life and had an average latency period of 10 and 25 days from birth following pre- and neonatal viral infections, respectively. The

<table>
<thead>
<tr>
<th>Group</th>
<th>Virusa</th>
<th>Day of pregnancy injection</th>
<th>No. of live births/no. of embryos given injections</th>
<th>Viability rate</th>
<th>Survival at the following wk of age</th>
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<tr>
<td>1</td>
<td>KiMSV</td>
<td>11</td>
<td>60/192</td>
<td>31.2</td>
<td>34/24/17/15/8</td>
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<tr>
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<td>32.0</td>
<td>22/20/19/18/11</td>
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<td>13</td>
<td>48/82</td>
<td>52.0</td>
<td>30/24/20/18/13</td>
</tr>
<tr>
<td>4</td>
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<td>14</td>
<td>114/138</td>
<td>83.0</td>
<td>92/64/43/31/9</td>
</tr>
<tr>
<td>5</td>
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<td>45.0</td>
<td>11/8/7/6/2</td>
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<tr>
<td>6</td>
<td>KiMSV</td>
<td>Newbornb</td>
<td>59/59</td>
<td>100.0</td>
<td>59/59/58/42/0</td>
</tr>
<tr>
<td>7</td>
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<tr>
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<td>45.0</td>
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<td>77.2</td>
<td>41/25/18/14/4</td>
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<td>48/49</td>
<td>98.0</td>
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<tr>
<td>12</td>
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<td>Newborn</td>
<td>25/25</td>
<td>100%</td>
<td>25/25/25/13/0</td>
</tr>
<tr>
<td>13</td>
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<td>11</td>
<td>47/53</td>
<td>89.0</td>
<td>43/43/41/37/31</td>
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<tr>
<td>14</td>
<td>Control</td>
<td>12</td>
<td>50/53</td>
<td>94.0</td>
<td>46/45/42/39/32</td>
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<tr>
<td>15</td>
<td>Control</td>
<td>15</td>
<td>30/30</td>
<td>100.0</td>
<td>28/28/28/27/24</td>
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</table>

a The approximate dose level injected was 1500 and 15 FFU/g of body weight for the KiMSV and MoMSV viruses, respectively.

b Newborn, within 24 h of life.

c Controls receiving the virus-free medium.
tumors grew in 2 distinct locations of the body, one at or near the injection site (abdominal or thoracic space) and the other at a distant site, specifically affecting the epithelial cells of the brain. Grossly the tumors arising in areas around the sites of injection were hard, gray-white nodules, 1 to 10 mm in diameter, attached to the inner sternum or between the ribs, scattered throughout the diaphragm, or distributed in various regions of the abdominal cavity. These tumors had a close morphological similarity to those described previously (21-24), and we classified them as mixed mesenchymal sarcomas.

There was a positive and highly significant ($P < 0.01$) association between the oncogenic potential of MoMSV and the stage of maturation affected, and while, for example, no sarcomas appeared in Group 7, they were induced in 71% of the pups of Group 9 and in 96% of those belonging to Group 12 (Table 2; Chart 1A) ($\chi^2$ for trend, 5.84; $P < 0.025$). By comparison, the KIMSV virus induced a low incidence of sarcomas (below 20%) at every developmental stage injected, with the exception of prenatal Day 14 where the exposure to the virus caused a 30% incidence of sarcomas in newborn. In this case there was no association between the day at treatment and the incidence of sarcomas ($\chi^2$ for trend, 0.51; $P > 0.05$). Several cell lines, established from these tumors, were shown to possess a malignant nature as demonstrated by their capability to grow and kill the hosts when reinjected into mouse fetuses and to form colonies in soft agar (data not shown).

The high and unexpected mortality occurring early during postnatal life, together with the insurgence of neurological signs (loss of equilibrium or partial paralysis) and of bulged cranium identified in several pups, suggested that the brain was a target of the injected viruses. Unfortunately, many of the potential brain tumor carriers were lost due to accidental events (cannibalism, decomposition, etc.), but the number of those examined is sufficiently representative of the pathological specificities induced by the MSV viruses. At autopsy, the brain surface appeared frequently punctuated with hemorrhagic spots, distributed along the cerebral hemispheres and/or the cerebellum. Upon dissection, cellular masses, soft and hemorrhagic, 3 to 10 mm in diameter, were observed. We have recognized 3 types of brain tumors, including tumors of the choroid plexus and capillary and cavernous angiomas. There was a significantly increased incidence ($P < 0.01$) of these neoplasms in treated, compared to control, animals. Although the percentage of affected pups was relatively high (approximately 40%) following the injection of MoMSV on Day 13 and of KIMSV on Day 15 of pregnancy, we found no association with the developmental day at treatment ($P$ for trend, $>0.05$) (Table 2; Chart 1B).

The tumors of the choroid plexus were detected only in animals treated at 11 to 13 days of pregnancy. Of 8 pups affected by this neoplasm, 2 had received the KIMSV virus, and 6, the MoMSV virus. They occupied the ventricular lumen and microscopically were composed of grossly papillary structures lined by a single layer of cuboidal or prismatic epithelial cells (Fig. 1). There were many bizarre cells inside the tumors, and mitotic figures were frequent.

Capillary angiomas were found in approximately 70% of the pups with brain tumors and appeared mainly in those animals which were exposed to the KIMSV and MoMSV viruses during Days 13 to 15 of pregnancy. Histologically the neoplastic proliferations affected the smallest blood vessels with the capillaries greatly increased in number and arranged haphazardly. The proliferating capillaries were several cellular layers thick and were composed of rounded polymorphic cells. Frequently larger vessels were involved, and tumoral cells appeared sometimes sprouting into the vascular lumen (Figs. 2 and 3). Samples of these tumors have been successfully transplanted into fetal mice and established as permanent lines in our laboratory. A low incidence of cavernous angiomas appeared in all the experimental groups considered. They were distinguishable from the other vascular tumors, because the blood vessels were enlarged and closely clustered together. These tumors were sometimes associated with capillary angiomas in the same brain.
exposed to the KiMSV and MoMSV viruses had vascular changes (Fig. 4). Pathological changes somewhat akin to those observed in the brains, including capillary telangiectases, endothelial abnormal proliferations of varied sites, and hemorrhagic areas of organs and tissues of the control animals. Such lesions were not found in the brain or other sites, i.e., infection and transformation of epithelial cells of the brain. The phenomenon is not species specific, and comparable results have now been obtained in Fischer rats (data from our laboratory), suggesting a virus-cell interaction event likely to depend on particular stages of cellular maturation and organization. In some respects our results compare with recent data that, in transgenic mice, the epithelial cells of the choroid plexus are a primary target of SV40-transforming genes introduced into the fertilized eggs by way of appropriate plasmids (31). The remarkable organotropism displayed by our viral inocula prompts the need to determine whether viral subpopulations and/or recombaints were present in the stock of MoMSV and KiMSV used. Since these viruses carry well-defined DNA sequences whose homologies are found in the normal cellular genome of a variety of organisms (32–37), it would also be of interest to look at the role of DNA methylation, a process of gene regulation known to vary between organs and tissues of developing organisms (38–40), as a candidate modulator of viral carcinogenesis in mammalian embryos.

One interesting aspect of transplacental carcinogenesis concerns the repeated observation that chemical carcinogens are usually unable to induce neoplasms in rodent embryos until organogenesis is near completion (5, 6, 41). To determine whether oncogenic stimuli induced by the addition of genetic material, as opposed to DNA disturbances caused by genotoxic chemicals, overcome the apparent resistance of midgestation embryos to cancer, we injected the MoMSV and KiMSV viruses...
into the gravid uterus at 11 and 12 days of pregnancy and found only a marginal incidence of tumors (below 20%) in the newborn, compared to an incidence of 55 to 80% when later stages of embryogenesis were exposed. Actually the effect was not due to failure of the injected viruses to infect the embryos because tissue damages including vascular abnormalities of the brain, liver necrosis, and splenomegaly were commonly found among the tumorous and nontumorous pups. The present data on the low incidence of tumors in mouse pups treated with MoMSV and KIMS at midgestation (Groups 1, 2, 7, and 8 of Table 2) bear some similarity with those described by others that 4-day-old, but not 14-day-old, chick embryos are completely resistant to sarcoma induction by the Rous sarcoma virus (42). On the other hand, there exists at least one further report indicating that the microinjection of the Moloney strain of murine leukemia virus into mouse embryos in utero at Days 8 or 9 of gestation resulted in leukemia development in about half of the newborn (12). Taken at face value, such controversial findings suggest that certain embryonal factors, essential to the emergence of organ and tissue primordia (38), control the expression and/or survival of transformed phenotypes derived from the injection of oncogenic retroviruses into mouse embryos during active organogenesis.

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REFERENCES

Fig. 1. Sarcoma of the choroid plexus observed in a 2-wk-old female CD-1 pup inoculated with MoMSV on Day 12 of embryonal growth. Many tumor cells appear elongated, spindle shaped or large and hyperchromatic. There is a gross papillary arrangement of the tumor, and a delicate fibriillary stroma is interspersed among cells. H & E, x 10.

Fig. 2. Capillary angioma of the brain induced in a 5-wk-old female CD-1 pup treated with KIMSV at 15 days of fetal development. The tumor is composed of a solid mass of capillaries greatly heterogeneous in dimension and with prominent endothelial lining cells. H & E, x 4.

Fig. 3. Capillary angioma of the brain induced in a 4-wk-old male CD-1 pup exposed to MoMSV during Day 15 of intrauterine life. The tumor shows proliferating capillaries which are several layers thick and in some areas appear tightly packed to give an almost uniform cellular mass. H & E, x 10.

Fig. 4. Pathological changes observed in the cerebral hemispheres of a 3-wk-old male CD-1 pup treated with KIMSV on Day 12 of embryonal development. Multiple thin-walled telangiectatic capillary vessels are present together with hemorrhagic areas and abnormal proliferation of endothelial cells. H & E, x 10.
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