Widespread Inhibition of Neuroblastoma Cells in the 13- to 17-Day-Old Mouse Embryo

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ABSTRACT

Previous experiments have demonstrated specific inhibition of tumor formation after neuroblastoma cells were injected into fragments of 8.5- to 9.5-day embryonic tissue (A. H. Podestà et al. Proc. Natl. Acad. Sci. USA, 81: 7608-7611, 1984). The effect was localized to the somite and appeared specific for neuroblastoma as opposed to a variety of other tumor types. This regulation of neuroblastoma cells was believed to reflect an underlying event in the development of migrating embryonic neuroblasts. The current experiments were done to determine the effect on regulation with further embryonic development. The results indicated that later embryos (13 to 17 days of gestation) have a widespread inhibitory effect in all tissues tested, including the adrenal gland, testis, kidney, liver, limb bud, and heart. In contrast a leukemia cell line was not affected by any of these tissues. In organ culture demonstrable colony formation by neuroblastoma was likewise inhibited, and conditioned media from one of these embryonic sources (limb bud) slowed but did not abrogate growth of neuroblastoma cells.

INTRODUCTION

Cells derived from neural crest migrate widely in the mouse embryo from the time of crest formation in the dorsal midline at 8.5- to 9.5 days gestation (1). Ultimately these migratory cells contribute to a variety of tissues. In particular, some of the cells migrate ventrally between the paired somites flanking the neural tube and ultimately contribute to the autonomic nervous system, including the adrenal medullae (2-5). Determination of neural crest cells has been studied by a variety of methods, still it is not clear what mediates the migration path, progressive restriction of developmental capability, or site of colonization in end organs. Some experiments point to an active role for the end points of migration (e.g., Ref. 6) whereas others point to early generation of subpopulations that might occur before migration (e.g., Ref. 7).

Our approach has been to use mouse neuroblastoma cells, derived from a widely studied transplantable tumor, C1300, as a probe of neural crest maturation. This approach was fostered by previous studies in which differentiation of tumor cells, either spontaneous or induced, has been closely accompanied by loss of tumor formation (8-12). Also, other tumor types, including teratocarcinoma and leukemia, have been shown to react with their corresponding developmental fields (13-20). The C1300 cell line of neuroblastoma used in our experiments can be induced to differentiate (21), and this is accompanied by loss of malignant potential (22). Thus, it was reasoned that if C1300 neuroblastoma were to respond to a developmental mechanism aimed at normal migrating neuroblasts, then there would be a concomitant loss of tumor forming ability.

The use of tumorigenicity as an assay has one clear advantage over other methods. The built-in amplification of tumor formation leads to a sensitive assay which, with appropriate controls, yields quantitative data (18). The disadvantage is that in isolated experiments it is not known whether cells which fail to form tumors do so because they have died due to technical reasons, because they have been killed specifically, or because they have been induced to terminally differentiate. Aside from routine controls for cell viability, we have relied on the demonstration of a pattern of specific regulation of tumor formation as an indicator that there may be an underlying developmental mechanism. Such a pattern might include specificity for time in development, for location in the embryo, and for the type of cancer cell used as a target.

Using this tumorigenicity assay with fragments of 8.5- to 9.5-day embryo we have previously reported that C1300 neuroblastoma responds to the region of the somite but not to the forming neural crest, the interior of the neural tube, the heart, or the brain (1). Likewise, exposure to fragments of adult liver does not affect tumorigenicity by C1300 neuroblastoma (1).

The current series of experiments were designed to test the effect of further somite development during organogenesis on the regulation of neuroblastoma.

To this end a variety of tissues from 13- to 17-day embryos were isolated and tested for their effect on tumor formation by neuroblastoma. Recognizing that most neuroblastomas arise in the adrenal medullae, a prime focus was the adrenal anlage at the time when it could be first detected and isolated at 13-14 days. At this time the normal migrating neuroblasts have been in the gland for approximately 24 h (23).

MATERIALS AND METHODS

Methods. The strategy of these experiments was an extension of those previously reported in which the effect of 8.5- to 9.5-day embryonic tissue or tumorigenicity by C1300 neuroblastoma cells was tested (1). In a typical experiment, 5 cancer cells were injected into fragments of embryonic tissue, and the fragments containing the neoplastic cells were transferred into adult mice in an appropriate site for subsequent tumor formation. The incidence of tumor formation was compared to that for 5 cancer cells transplanted into the same site in the absence of embryonic tissue. The adult hosts were syngeneic with the tumor cells, whereas the hosts were allogeneic with the embryonic tissues. As previously shown, the embryonic tissue undergoes homograft rejection without interfering with tumor formation by the cancer cells (17). The L1210 leukemia cell line was used as a control in these studies; its lack of regulation by a variety of embryonic tissues (1, 18) ensured that loss or damage to the small numbers of cells used in these experiments was not responsible for any loss of tumorigenicity.

Cells and Tissue Culture Conditions. The subline of C1300 used in these experiments was obtained from Prasad (21), maintained by serial culture in Ham's F-12 plus 10% fetal calf serum (non-heat denatured fetal calf serum) at 37°C in a 5% CO2 atmosphere, and tested for tumorigenicity in A/J mice (The Jackson Laboratory) by transplantation into the testis. Neuroblastoma cells for experiments were prepared by treatment with 2.5% trypsin for approximately 30 s; the trypsin was removed and the cells were resuspended in cloning medium (Ham's MCDB) supplemented with 5% fetal calf serum (23). L1210 leukemia cells were serially transplanted as an ascites tumor in DBA/2 mice (The Jackson Laboratory), washed 3 times from the ascites fluid by slow
speed centrifugation, and resuspended in RPMI + 5% fetal calf serum as reported previously (18).

Embryonic Tissues. Mouse embryos at 13 to 17 days gestation (day 1 was the day when a vaginal plug was observed) were removed aseptically from the uterus and the organs were dissected under a Nikon dissecting scope. Organs were identified by their anatomic relationships (24) and gross appearance. Adrenal glands could first be seen with the aid of a dissecting microscope, isolated by dissection, and confirmed histologically at 13 days of gestation. Other retroperitoneal organs, which condensed at the same time and were roughly the same size, included kidney and testis. The identity of several samples of isolated organs was confirmed histologically after fixation and paraffin embedding. Whole adrenals, testis, and kidneys were used. Limb buds or digits thereof were cleanly amputated for use. Comparably sized cubes of liver, lung, and heart muscle (roughly 1 mm in dimension) were obtained from isolated tissues by dissociation in 2.5% trypsin for 20 min. Cells from 5–10 tissue fragment wells were aliquoted into individual wells of Linbro plates (Falcon) with 0.2 ml of medium. Conditioned medium was collected at 24–48 h from confluent monolayers.

Injection of Neuroblastoma Cells into Embryonic Fragments for Tumor or Colony Assay. This was done as described previously (1). Isolated embryonic fragments were pooled in an operating drop of cloning medium under paraffin oil. Cancer cells were placed in a separate donor drop. By means of a Leitz micromanipulator, 5 cells were aspirated into an injecting pipet, the tip of which was slightly larger than 1 cell diameter. The cancer cells were then moved to the operating drop where they were injected into the embryonic tissue (held by a second larger pipet) under direct visual control along with a few cell volumes of medium. Successfully injected fragments were transplanted into anesthetized adult hosts (experimental group); as controls, unused cancer cells from the donor drop were transplanted in an identical manner. Transplantation was done with finely drawn, orally controlled pipet under direct visual control and with microliter amounts of fluid. Animals were observed for at least 2 months and tumors were examined histologically. To assay growth in vitro, similar techniques were used, except that specimens were transferred to individual wells of Linbro plates containing 0.2 ml of medium and observed for subsequent growth microscopically (20). The results of tumor or colony assays were expressed on the basis of whether an animal or a tissue culture well contained an identifiable focus of cancer cells. The ratio of positive specimens to total tested was compared between experimental and control groups using the Mantel and Haenszel modification of the $\chi^2$ test (25). For comparison of colony areas, colony diameters were measured with an ocular micrometer, areas were calculated as a circle, and the data were analyzed with the Student $t$ test.

RESULTS

Technical Validity of the Tumor Assay. To ensure cell accountability (i.e., that the small numbers of cells were not lost or killed by the manipulations), 5 leukemia cells were injected into tissue fragments and the incidence of tumors was compared to that obtained with 5 cells in the absence of embryonic tissue. Leukemia cells were chosen because they produce tumors rapidly and have never shown regulation in previous experiments. As shown in Table 1 there was no significant difference between experimental and control groups. This was in accord with previous experience with similarly sized tissue fragments and a variety of tumors, including neuroblastoma (1).

Tumorigenicity of Neuroblastoma Cells Exposed to Various Embryonic Tissues. As was done with the leukemia cells, 5 C1300 neuroblastoma cells were also injected into each embryonic fragment, and the incidence of tumors was compared to that obtained with 5 control cells in the absence of embryonic tissue. As shown in Table 2, all tissues tested from 13–14-day embryos as well as from 16–17-day embryos markedly suppressed tumor formation by neuroblastoma cells. This included not only the obvious target of neuroblast migration, the adrenal gland, but other retroperitoneal organs (kidney and testis), liver, lung, heart muscle, and digits of the limb buds.

Colony Formation by Neuroblastoma Cells Exposed to Embryonic Tissues in Organ Culture. In attempts to reproduce the effect on growth in vitro, embryonic fragments each treated by injection of 5 neuroblastoma cells were placed in individual tissue culture wells. After 1–2 weeks in culture, in some cases the neuroblastoma cells had grown to become clearly identifiable colonies of tumor cells against the background of proliferating embryonic cells; these wells were scored as positive for colony formation. In other cases neuroblastoma cells failed to proliferate even during an additional 2-week period with refeeding; these were scored as negative for colony formation. Within the framework of these operational definitions it was clear that colony formation in the presence of embryonic tissues (adrenal, testis, and kidney) was reduced as compared to that for cells cultured in the absence of embryonic tissues (Table 3). Similar results were obtained for coculture of neuroblastoma cells on monolayers of cells from dissociated embryonic organs (data not shown).

Effect of Media Conditioned by Embryonic Tissues on the Growth of Neuroblastoma Cells. Medium was obtained from confluent primary cultures of embryonic organs diluted to a final concentration of 20% in fresh growth medium and tested for its effect on colony formation by neuroblastoma cells. The same lot of medium, left unexposed to tissue, was used as the control. As shown in Table 4, there was no significant effect on the rate at which 5 neuroblastoma cells in a tissue culture well would form a colony. This was true for conditioned media from adrenals, testis, kidney, and limb bud digits. However, the colonies appeared to contain fewer cells after 1 week of growth. This was confirmed using limb bud conditioned media. The diameter of individual neuroblastoma colonies growing either in 20% conditioned media or growth media was measured with an ocular micrometer, and colony area was calculated. As shown in Table 5, each of 3 independent lots of conditioned media reduced growth rate of neuroblastoma cells. In these experiments individual cells in limb bud conditioned media again formed colonies (45/105 (43%) at a rate similar to that for controls (62/120 (52%); the difference is not statistically significant. The underlying assumption was that colony area would be proportional to cell numbers. This seemed reasonable since differences in cellular and colony morphology were slight between treated and untreated groups. Under this assumption, the data in Table 5 show a 3–7-fold reduction in cell number after 1 week of growth in 20% limb bud conditioned media.

DISCUSSION

In previous studies with tissues from somite-stage embryos (days 8 to 9 of gestation) isolated somites had a specific inhibi-
EMBRYONIC INHIBITION OF NEUROBLASTOMA

Table 2 Results of tumor assay with NBP2 neuroblastoma cells exposed to embryo fragments

<table>
<thead>
<tr>
<th>Embryonic organ</th>
<th>Experimental result (no. of tumors/no. of transplants)</th>
<th>5 cell alone controls (no. of tumors/no. of transplants)</th>
<th>α</th>
</tr>
</thead>
<tbody>
<tr>
<td>13-14-day adrenal</td>
<td>6/46 (13)*</td>
<td>44/52 (85)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>16-17-day adrenal</td>
<td>8/37 (22)</td>
<td>31/41 (76)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>13-14-day testis</td>
<td>6/36 (17)</td>
<td>33/36 (85)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>16-17-day testis</td>
<td>9/38 (24)</td>
<td>36/39 (92)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>13-14-day kidney</td>
<td>0/26 (0)</td>
<td>23/29 (79)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>13-14-day liver</td>
<td>2/27 (5)</td>
<td>25/45 (56)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>13-14-day heart</td>
<td>2/12 (17)</td>
<td>12/15 (80)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>13-14-day limb bud digits</td>
<td>9/36 (25)</td>
<td>28/32 (88)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>13-14-day lung</td>
<td>7/27 (26)</td>
<td>17/29 (59)</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage.

Table 3 Results of colony assay with NBP2 neuroblastoma cells exposed to 13-14-day embryo fragments in organ culture

<table>
<thead>
<tr>
<th>Embryonic organ</th>
<th>Experimental result (no. of positive wells/total)</th>
<th>Control results (no. of positive wells/total)</th>
<th>α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal glands</td>
<td>25/58 (43)*</td>
<td>78/80 (97)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Testis</td>
<td>2/23 (9)</td>
<td>40/40 (100)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Kidney</td>
<td>1/21 (5)</td>
<td>39/40 (98)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage.

Table 4 Effect of conditioned media from 14-day embryonic organs on colony forming efficiency by 5 neuroblastoma cells

<table>
<thead>
<tr>
<th>Source of conditioned medium</th>
<th>Experimental (no. of positive wells/total)</th>
<th>Growth medium control (no. of positive wells/total)</th>
<th>α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal glands</td>
<td>18/21 (86)*</td>
<td>22/30</td>
<td>NS*</td>
</tr>
<tr>
<td>Testis</td>
<td>15/18 (83)</td>
<td>22/30</td>
<td>NS</td>
</tr>
<tr>
<td>Kidney</td>
<td>13/23 (57)</td>
<td>22/30</td>
<td>NS</td>
</tr>
<tr>
<td>Limb bud digits</td>
<td>22/23 (96)</td>
<td>22/30</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage.

Table 5 Effect of limb bud (LB) conditioned media on the mean area of neuroblastoma colonies

<table>
<thead>
<tr>
<th>Medium batch</th>
<th>Experimental with 20% LB conditioned media</th>
<th>Control with 20% growth media</th>
<th>α</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>157</td>
<td>1072</td>
<td>0.01</td>
</tr>
<tr>
<td>2</td>
<td>233</td>
<td>501</td>
<td>0.01</td>
</tr>
</tbody>
</table>

9 and 14 days of gestation, somites will contribute to the mesenchyme of a variety of structures during organogenesis, and organ specific mesenchymal-epithelial interactions have profound effects on morphogenesis (6, 27, 28). It seemed possible that what we originally measured was not the effect of newly formed somites, but rather the effect of more mature, organ specific mesenchyme derived from them.

To investigate this possibility, the anlage of various organs were tested for their effect on neuroblastoma. As reported here, a wide variety of tissues (isolated between 13 and 17 days of gestation) had dramatic inhibitory effects on tumor formation by neuroblastoma cells. This included two tissues, heart and liver, which although already formed had not been inhibitory at the somite stage of development. In particular, no organ specific pattern of inhibition was discerned; many tissues inhibited neuroblastoma as effectively as the adrenal primordium.

If the mechanism of inhibition by later embryonic tissues (14- to 17-day) is the same for each tissue and the same as for 9-day somites, then one might postulate a widespread mechanism related to developing somatic mesenchyme that is switched on between 10 and 13 days of gestation. In later embryos, this might have the effect of globally suppressing growth of cells which have not reached a proper end organ. It might also mask organ specific effects on cancer cells.

Support for these ideas comes from a variety of observations. For instance, it would seem that not all migratory cells reach the appropriate organ, and these misplaced cells then usually fail to grow to a significant extent. For example, primitive germ cells migrate from the region of the yolk sac to colonize the gonad, but some germ cells may be left in the dorsal midline. Transformation of such ectopic cells could be an explanation for extragonadal germ cell tumors. In addition, late stage embryos have been said to be a difficult site for tumors to grow in (29). Finally, soluble substances with potent activities on cell growth may be acting during organogenesis. Later embryos have been a rich source of -transforming growth factor, a substance that inhibits growth of many tumors including melanoma (30). Epidermal growth factor levels are markedly reduced after 14 days of gestation (31). Pertinent to this sort of argument is the observation that normal embryonic sympathetic neuroblasts are dependent for growth in culture on a partially purified factor (CMF) and rapidly become dependent on NGF (32). Trophic substances such as these may play a role in the programmed cell death that occurs during nervous system development (33) and selective cell death may be as important to understand as differentiation.

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3 Unpublished data.
Growth inhibitory activity for neuroblastoma is present in media conditioned by the limb bud. In tissue culture the effect is primarily seen as slowed growth rate rather than total cessation of growth, and it is not yet clear whether depletion of growth factors or addition of inhibitory factors is responsible. This same limb bud conditioned medium has a similar effect on another neural crest derived tumor (melanoma). In the case of melanoma the ability of embryonic skin tissue to provide inhibitory conditioned media is correlated with the time of arrival of normal melanoblasts into area of skin. It is possible that tissue specific effects will be masked by global inhibitory effects at some stages of development.

REFERENCES

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