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The neurula stage mouse embryo in control of neuroblastoma

(neoplasia/regulation/tumor)

AUGUSTO H. PODESTA, JUNE MULLINS, G. BARRY PIERCE*, AND ROBERT S. WELLS

Department of Pathology, University of Colorado Health Sciences Center, 4200 East Ninth Avenue, Denver, CO 80262

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ABSTRACT **The purpose of this study was to determine whether the neurula stage mouse embryo can regulate tumor formation of C-1300-3 neuroblastoma cells. Five neuroblastoma cells were injected into the second somite of neurula stage embryos, and their ability to form tumors was tested, 24 hr later, by transplanting the portion of the embryo containing the cancer cells into the testes of adult mice. Only one-third the number of tumors was obtained in comparison with controls in which (*i*) five neuroblastoma cells were injected into blocks of liver tissue that were then transplanted into the testes of adult animals or (*ii*) five C-1300-3 neuroblastoma cells were injected directly into the testes. When five C-1300-3 cells were injected into somites, which had been dissected from embryos, and the injected somites were placed in animals, significantly fewer tumors were obtained in relationship with controls. Although it is not known whether the neuroblastoma cells are induced to differentiate or are killed by the embryonic tissue, the effect appeared to be specific because the tumor-forming ability of L1210 leukemia, B-16 melanoma, embryonal carcinoma 247, and a parietal yolk sac carcinoma was unaffected by somites.**

Cancer cells can differentiate into benign if not normal cells: observations were made first with teratocarcinomas (1–3), and subsequently have been made with neuroblastoma (4, 5), leukemias (6, 7), squamous cell carcinomas (8), and adenocarcinomas of the breast and colon (9). Differentiation has been induced in tumors by irradiation (10), chemicals (11), analogues of vitamins (12), and growth factors that control physiologic responses of normal hematopoietic and leukemic cells (13). Clearly, direction of the naturally occurring differentiation of neoplasms could result in a noncytotoxic treatment for cancer (14).

Important in this regard is the observation that the mouse blastocyst can regulate embryonal carcinoma cells to the point that they and their offspring take part in normal embryonic development leading to the production of chimeric mice (15–19). In an attempt to understand the mechanism of this regulation, an assay, based on the ability of the blastocyst to regulate tumor formation of embryonal carcinoma cells, was developed (20). In this assay, the incidence of tumors obtained when single embryonal carcinoma cells were injected into suitable animals was compared with that when the cancer cells were incorporated into blastocysts that were then placed in the same site in the animal. Two of three embryonal carcinomas tested produced tumors in significantly fewer numbers than in control situations, and the reaction had a degree of specificity because the tumor-forming ability of B-16 melanoma cells, sarcoma 180 cells, and L1210 leukemias was not affected by the blastocyst (21). A small but significant degree of abrogation of tumor formation was always observed when C-1300-3 neuroblastoma cells were tested in the assay. This was an unexpected observation because C-1300-3 neuroblastoma cells, like B-16 melanoma cells and L1210 leukemia cells, have no cellular counterpart in the blastocyst. Neuroblastoma cells are derived from the neural crest, which develops about 4 days after the blastula. Possibly the neuroblastoma cells were regulated by the embryo as it neurulated (21). The purpose of this study was to determine whether or not the neurula stage embryo can abrogate tumorigenicity of C1300 neuroblastoma cells.

MATERIALS AND METHODS

The plan of the experiment was to inject C-1300-3 cells into the somitic mesoderm of neurula stage mouse embryos, culture the embryos *in vitro* for 24 hr, and then transfer the part of the embryo with the cancer cells to adult hosts isologous with the cancer cells but homologous with the embryonic cells. The embryo was destroyed in 6–9 days by the ensuing homograft rejection (a technique used successfully in the assay for blastocyst control of tumor formation of embryonal carcinoma) (20). The cancer cells, isologous with the host, survived and formed tumors unless their tumor-forming ability was adversely affected by the embryonic tissues. To this end the number of tumors obtained in the experimental group was compared with those of controls in which the same number of cancer cells was injected into blocks of liver tissue isologous with the embryo and of the same size as the part of the embryo employed. These blocks of liver were then injected into the homologous hosts. The incidence of tumors produced in this control corresponded so closely to the incidence of tumors obtained when the cancer cells were injected directly into the hosts that in later experiments tumor cells were injected into the testis as the control. In other words, the homograft reaction to the liver tissue or embryonic tissue did not so damage the C-1300-3 cells that they could not form tumors in numbers equal to those of the other controls.

The tumors employed in this study are listed in Table 1 with their general characteristics.

Embryos of $8\frac{1}{2}$ days of gestational age (day 1 was the day when a vaginal plug was observed) were dissected from the uterus using a dissecting microscope, watchmaker's forceps, and Hanks' balanced salt solution/10% fetal calf serum. The visceral yolk sac and ectoplacental cone were left intact, but Reichert's membrane with attached trophoblast was removed. It was possible to focus through the visceral yolk sac and identify the embryo and its several parts, but the visualization of the somites was less than optimal. Suspensions of tumor cells were obtained using standard trypsinization techniques and after testing for viability by trypan blue exclusion were placed with an appropriate number of embryos in a drop of medium under mineral oil. All tissue cultures were fed Ham's F-12 medium/10% fetal calf serum containing antibiotics and were incubated at 37°C.

The techniques used for injection of embryos with cells were similar to those described previously (20, 21). Embryos were oriented on a holding pipette by gentle suction and five neuroblastoma cells were placed in the second somite on the

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*To whom reprint requests should be addressed.

Table 1. Lines of tumor

Tumor/source	Host strain	Method of propagation	Cloning site (efficiency)	Comments
C-1300-3 (neuroblastoma)/ The Jackson Laboratory	A/J	Subcutaneous	Testis (40–50%), *in vitro* (40–70%)	Solid tumor, grown *in vitro* for 16 hr prior to experiment
NBP-2 (neuroblastoma)/ Prasad *et al.* (22)	A/J	Tissue culture	Testis (86%)	Adapted for tissue culture studies
EC 247 (embryonal carcinoma)/Lehman *et al.* (23)	129	Monolayer tissue culture	Testis (30%)	Maintained by passage every 48 hr, multipotent but undifferentiated
L1210 (leukemia)/animal/ human tumor bank	DBA/2	Ascites	Intraperitoneum (25–35%)	Ascites
B-16 (melanoma)/Stackpole (24)	C57/BL6	Monolayer or *in vivo*	*In vitro* (30%)	Solid amelanotic tumor readily produces *in vitro* monolayer cultures
PYS (parietal yolk sac carcinoma)/Pierce *et al.* (21)	129	Ascites	Intraperitoneum (20–30%)	Ascites
Sarcoma 180/animal/human tumor bank	DBA/2	Ascites	Testis (20–25%)	Ascites

right side of each embryo using a micropipette attached to a Leitz micromanipulator. Embryos were cultured *in vitro* for 24 hr using the medium devised by New (25) and Sadler and New (26) and the techniques of Beddington (27). Briefly, five embryos were transferred to a stoppered flask containing 2.5 ml of rat serum that had been inactivated at 56°C for 30 min before use. The flasks were gassed initially and again at 18 hr with 5% CO_2/95% air. They were rotated at 20 revolutions per min in a warm room at 37°C (26). All cultures were terminated at 24 hr, and the region of the second somite from each embryo bearing the cancer cells (roughly the trunk region of the embryo) was transferred to the left testes of mice of the appropriate strain to test whether the cancer cells could form tumors. Control animals received either five C-1300-3 cells in the left testis or five C-1300-3 cells that had been injected into blocks of CD-1 liver tissue of similar size as the portions of the embryos that were transplanted. The blocks of liver tissue were placed immediately in the testes. Animals were examined weekly for 8 weeks for tumors. All tumors were examined microscopically. The data were analyzed according to the method of Mantel and Haenszel (28), which is a modified χ^2 test.

To confirm the data, a method that provided better visualization of somites and more accurate placement of tumor cells was used. Day 9½ mouse embryos were removed from the proximal endoderm and the trunk of the embryo was cut transversely into segments, each five somites long. The middle somite on one side of the segment was injected with five cells and the injected segment was put immediately into the testis of an adult animal as described above. Segments were used from the anterior and posterior end of the embryo (oldest and youngest somites) to see whether somitic age influenced the outcome of the experiments. For a control, five neuroblastoma cells were injected into the testis. Cells were not lost from these segments because they could be recovered quantitatively in tissue culture from segments lethally irradiated prior to injection.

To begin study of the mechanism of regulation of tumor formation by the embryo, five C-1300-3 cells were injected into somites, many of which could be dissected from a single CD-1 embryo. To this end, day 8½–9 embryos were placed in a drop of 0.025% pancreatin until the tissues began to loosen. The enzyme solution was replaced with tissue culture medium and the somites were dissected from the embryos with glass needles using a dissecting microscope. In effect, the neural tube was removed first and then the somites. Some overlying epidermal and other adjacent cells probably adhered to the somites. The somites were injected with tumor cells, as described above, and were put immediately into the testes of A/J mice. In the controls, five C-1300-3 cells were injected directly into the testes of A/J mice. The animals were then examined for development of tumors and autopsies were performed on all animals surviving for 60 days.

In an effort to determine whether there was embryologic specificity for this abrogation of tumor formation by the neurula stage embryo, L1210 leukemia, B-16 melanoma, EC 247 embryonal carcinoma, and parietal yolk sac carcinoma cells were injected into CD-1 somites and tumor formation was measured in the appropriate strains of mice as described above.

RESULTS

In 80% of cases, day 8½ CD-1 mouse embryos injected with five C-1300-3 cells in the region of the second somite and then cultured for 24 hr attained development comparable with that seen by others for day 9 *in utero* embryos (27, 28). Somites increased from 4 or 6 to about 23 pairs, and beating hearts, yolk sac circulation, closure of neural tubes, and development of pharyngeal arches were observed. Completed axial rotation occurred. The 20% of cases that did not show this degree of development were discarded because it was possible that abnormal growth of the embryo might affect the growth of the cancer cells.

Forty-nine embryos grafted with C-1300-3 cells were used. Only 5 of them (10%) produced tumors on transplantation into the testes. This was significantly less than for control mice, which received five C-1300-3 cells, intratestis, and developed tumors in 35 of 52 animals (67%). When five C-1300-3 tumor cells were injected into blocks of liver tissue from CD-1 mice and immediately transferred to the testes of A/J mice, tumor formation was comparable with cases in which cancer cells were injected alone; 11 of 17 animals developed tumors (65%). This indicated that the homograft rejection of the embryonic tissue did not affect the growth of C-1300-3 cells, which were isologous with the host.

It was conceivable that some of the tumor cells might have been lost because of the necessity of keeping the proximal endoderm intact, thereby reducing visibility. To control this possibility, a line of embryonal carcinoma, F-9, that is not regulated by the blastocyst and theoretically should not be

regulated by the neurula was employed. Ten of 52 (19%) of F-9 cells (five cells per embryo) grafted into the second somite produced tumors when transplanted into strain 129 mice. Twenty-six of 62 (42%) of animals injected with five F-9 cells into the testis developed tumors. This suggested the possibility that 50% of tumor cells injected under the circumstances of the experiment may have been lost because of suboptimal visualization of the somites. When the data were corrected for that possibility, rather than the factor of 6 reduction in tumor formation of neuroblastoma there may have been only a factor of 3 reduction. Even so, this result is highly significant.

Most tumors developed within 30 days of injection. Tumors that developed from embryo segments containing cancer cells were always neuroblastomas indistinguishable from control tumors. Testes of animals that were injected but did not develop tumors were often atrophic and scarred. No neuroblastoma cells were found in those that were examined microscopically.

For the confirmatory experiment, it was decided to eliminate the tissue culture step, use a different substrain of C-1300 neuroblastoma cells that grows well in tissue cultures, obviate the optical problem, and determine whether the intact embryo was required for the regulation. To this end, five NBP-2 cells were injected into segments of the embryo and the injected segments were placed directly into the testes. There was an insignificant difference in incidence of tumors obtained when anterior or posterior embryo fragments were used and the data were combined. Thirty-three of 58 transfers (57%) developed into tumors in comparison with 57 of 66 controls (86%). The data are significant at the 0.01% level (28). Also, 22 of 46 (48%) of C-1300-3 cells injected into embryo segments gave rise to tumors in comparison with 36 of 39 (92%) of controls (significant at the 0.01% level).

For the final experiment, it was decided to test whether or not somites dissected from day $9\frac{1}{2}$ embryos might mediate the response. The rationale for the experiment was the observation that differentiation of neural crest cells occurs in the presence of somitic tissue. Five C-1300-3 cells were injected into freshly isolated somites and these somites were immediately injected into the testes of animals to test for tumor-forming ability of the tumor cells. Tumors were obtained in significantly fewer numbers than in controls (Table 2). To test for specificity of this reaction, L1210 leukemia, B-16 melanoma, EC 247, and PYS carcinoma cells were injected into somites and tested for tumorigenicity. These tumors grew as well after incorporation into somites as they did when injected into the testes in the absence of somites (Table 2). Thus it was concluded that there was a degree of specificity to the abrogation of tumor formation by somitic mesoderm of the neurula stage embryo.

DISCUSSION

Experiments in which neuroblastoma cells were injected into intact neurula stage embryos, segments of those embryos, or somites dissected from them have demonstrated a significant reduction in the ability of the neuroblastoma cells to form tumors. In addition, a degree of specificity for the effect is suggested by the observation that the tumor-forming ability of L1210 leukemia cells, embryonal carcinoma cells (EC 247), and parietal yolk sac carcinoma cells was not affected by somitic mesoderm from the neurula. This leads to the postulate that, if two embryonic fields can regulate their closely related tumors, in all probability there will be an embryonic field capable of regulating each type of cancer (29).

Table 2. Effect of somites on ability of malignant cells to form tumors

Tumor	Tumors from cells injected into somites, no. tumors/no. attempts	Controls, no. tumors/ no. attempts	Significance level*
C-1300-3	23/49 (47%)	38/56 (68%)	95%
EC 247	23/48 (48%)	23/49 (47%)	NS
PYS	21/54 (39%)	15/46 (32%)	NS
L1210	7/30 (23%)	4/29 (14%)	NS
B-16	19/52 (37%)	18/50 (36%)	NS

*By method of Mantel and Haenszel (28). NS, not significant.

In further support of this idea is the observation by Gootwine *et al.* (30) that leukemia cells injected into the placentas of day 10 mouse embryos did not produce leukemia in 2 of 140 animals injected. Rather, those 2 animals had leukocytes with the biochemical marker of the leukemia line. Apparently, in those 2 animals, the leukemia stem cells were induced to differentiate, possibly by the circulating regulators of leukopoiesis described by others (6, 7, 13) or by direct cell–cell contact. It is known that regulation of embryonal carcinoma by the blastocyst requires cell–cell contact (29). Understanding of the mechanisms of differentiation, whether in normal or neoplastic situations, could lead to specific, noncytotoxic forms of therapy (14).

It must be stressed that the mechanism of regulation of tumor formation of neuroblastoma cells has not been identified. Either the malignant cells are induced to differentiate (as in the chimeric mice produced by injecting embryonal carcinoma cells into blastocysts) as reported by others (15–19) or the cells die or are killed. Our unpublished data on the regulation of colony formation of embryonal carcinoma by the blastocyst are compatible with the idea that the embryonal carcinoma cells are induced to differentiate. Thus, differentiation will likely prove to be the mechanism of control of these neuroblastoma cells.

There are no precedents in the neuroblastoma literature for these experiments. C-1300-3 neuroblastoma is presumably derived by carcinogenesis of cells of the adrenal medulla, which are of neural crest origin. Neural crest cells develop in the posterior portion of the neural tube and commence migrating when the neural tube separates from the overlying ectoderm. Initially, trunk neural crest cells lie in the glycosaminoglycan-rich space bound by ectoderm, neural tube, and somites. Then they migrate laterally and form two streams separated by the somites. The lateral stream between the somite and ectoderm forms the pigment cells of the skin; the medial one between the somite and the neural tube forms the autonomic nervous system. The elegant studies of LeDouarin (31), Weston (32), and Thiery *et al.* (33) have shown that crest cells are highly motile and invasive and the environment of the migrating cells can in some cases specify what the differentiated phenotype of the migrating cells will be. Ganglion cells cultured in the environment of the neural crest migratory pathway of younger embryos can change their phenotypic expression from cholinergic to sympathetic cells. The tissues at the destination of the migrating cells may also be inductive of this differentiation (34).

For these reasons, we chose to study the effects of isolated somites for possible regulation of tumor formation. The data indicate that somitic mesenchyme has a regulatory effect on neuroblastoma formation, but the degree of regulation was significantly less with isolated segments of embryos than with intact embryos. Thus, it is possible that the intact embryo may have contributed something in addition to the effect of the somites or the somites may be the regulatory tissue but were not sufficiently developed to be maximally effective at the time chosen for these experiments. Norr (35) has tested the ability of the neural tube and somites to induce differentiation of neural crest cells into pigment producing cells *in vitro*. Both immature somites and neural tubes were able to induce some pigment formation of neural crest cells, but somites conditioned by neural tube were strongly induc-

tive. Finally, molecules of the ground substance may play an important role in the mechanism.

Thus, it is clear that many possibilities remain to be tested. One of the most promising will be to test the adrenal anlagen for possible inductive effects.

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