Direct Cloning of Human Neuroblastoma Cells in Soft Agar Culture

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ABSTRACT

An in vitro soft agar technique was used in an attempt to culture neuroblastoma cells from 71 bone marrow, 3 lymph node, and 2 solid tumor specimens from 18 patients with neuroblastoma. One-half of each specimen was sent for routine pathology studies and one-half was cultured in the soft agar system. Colonies appeared within 10 days in histologically positive bone marrows. Light microscopy, electron microscopy, catecholamine secretion, and karyology provided evidence that the colonies were composed of neuroblastoma cells. There were 38 instances in which histological study of the specimen demonstrated neuroblastoma cells. The soft agar system showed colony growth in 30 of these samples (79%). There were a total of 38 specimens that were histologically negative for neuroblastoma. Thirty of these 38 specimens showed no growth in the stem cell assay. Eight histologically negative specimens from 6 patients formed colonies in the soft agar system. Five of these 6 patients showed tumor histologically on prior or subsequent marrow examinations. In addition to a significant correlation between histological and soft agar culture results (p < 0.001), there exists a highly significant positive correlation between the number of colonies per plate and the histological status of the specimen (p < 0.005). Serial marrow samples were cultured on 7 patients. There appears to be an association between the number of colonies that develop in the plate and the clinical course and prognosis of the patient. Decreasing plating efficiencies (number of colonies per number of cells plated) correlated with tumor response. Increasing plating efficiencies indicated tumor relapse. A plating efficiency of ≥0.1% portended a particularly poor prognosis. Neuroblastoma grows well in this soft agar culture system. This excellent growth provides a good model for both clinical and basic science studies of neuroblastoma.

INTRODUCTION

Neuroblastoma is a common childhood cancer third in incidence only to leukemia and brain tumors. Unfortunately, despite advanced treatment methods that have proved successful in other childhood cancers, the long-term prognosis remains poor for patients with neuroblastoma. One problem is that 50 to 70% of the children who present with neuroblastoma have disseminated disease. Also, there are few drugs of known effectiveness against this cancer (12).

The purpose of the following study was to utilize a recently developed soft agar culture system in an attempt to grow neuroblastoma from a variety of clinical sources. This soft agar system has been reported to be selective for tumor growth (5–7, 23). In addition, it has been shown to be useful as a predictor for response of an individual patient’s tumor to chemotherapy (18, 19).

We now report the use of this soft agar system for the study of neuroblastoma. This paper describes the growth of neuroblastoma cells in the system, as defined by morphology, karyology, and secretion of a tumor marker. In addition, potential applications of this direct culturing technique for diagnosis, prediction of response, and prognosis of the patient with neuroblastoma are presented.

MATERIALS AND METHODS

Patient Population. Only patients with pathologically documented neuroblastoma were chosen for this study. The St. Jude’s clinical staging system was used (4). From October 1978 to November 1979, 18 patients with neuroblastoma were treated at 3 institutions participating in this study. At the time of diagnosis, 11 patients were Stage III C, 2 were Stage III A, 3 were Stage II B, and 2 were Stage IIA. The median age was 2.5 years with a range of 4 months to 11 years. There was only one patient less than 1 year old. All the patients had abdominal primary tumors. The sex distribution was equal. Bone marrow involvement was documented pathologically at the time of diagnosis in 11 of these patients.

After obtaining informed consent according to federal regulations, a total of 71 bone marrow aspirations (approximately 2.0 ml each) were performed on these 18 patients at various time intervals. Tissue was obtained by open biopsy from lymph nodes in 3 patients, primary tumor in 1 patient, and open liver biopsy in 1 patient. These procedures were all obtained as part of routine diagnostic and follow-up studies. One half of each specimen was sent for routine pathology studies, and the other half was sent at ambient temperatures from 2 of the investigators’ hospitals (those of J. Casper and A. Altman) to the stem cell laboratory of the principal investigator (D. D. Von Hoff). These specimens were sent blindly without clinical information or results of pathological examination of the aspiration or biopsy specimen known to the principal investigator. The average delay from sample collection to placement in culture was 48 hr. At the end of the study, patient identification was unblinded, and pathological, clinical, and stem cell correlations were performed.

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Collection of Cells. Bone marrow cells were obtained by iliac crest puncture. Cells were aspirated into a syringe containing preservative-free heparin (100 units/ml). After centrifugation at 150 \( \times \) g for 10 min, the cells in the buffy coat were harvested with a Pasteur pipet and washed twice in Hank’s balanced salt solution (Grand Island Biological Co., Grand Island, N. Y.) with 10% heat-inactivated fetal calf serum (Grand Island Biological Co.). Lymph nodes or other tissues obtained immediately after surgery were mechanically dissociated under aseptic conditions. These solid tissues were minced with a scalpel, teased apart with needles, passed through 20-, 22-, and 25-gauge needles and then washed by centrifugation as previously described (7). The viability of both bone marrow and lymph node specimens was determined in a hemocytometer with trypan blue. Viability after shipment in the mail for 48 hr was routinely more than 90% for marrow specimens and 4 to 70% for lymph node and solid tumor specimens.

Culture Assay for Tumor Colony-forming Cells. Cells were cultured as described by Hamburger and Salmon (5, 6). Cells to be tested were suspended in 0.3% agar in enriched CMRL 1066 medium (Grand Island Biological Co.) supplemented with 15% horse serum, penicillin (100 units/ml), streptomycin (2 mg/ml), glutamine (2 mm), \( \text{CaCl}_2 \) (4 mm), and insulin (3 units/ml). Just prior to plating, asparagine (0.6 mg/ml), DEAE-dextran (0.5 mg/ml; Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), and freshly prepared 2-mercaptoethanol (final concentration, 50 \( \mu \)M) were added to the cells. One ml of the resultant mixture was pipetted onto 1-ml feeder layers in 35-mm plastic Petri dishes. The final concentration of cells in each culture was 5 \( \times \) 10^6 cells in 1 ml of agar:medium. The feeder layers used in this study consisted of McCoy’s Medium 5A plus 15% heat-inactivated fetal calf serum and a variety of nutrients as described by Pike and Robinson (14). Immediately before use, 10 ml of 3% tryptic soy broth (Grand Island Biological Co.), 0.6 ml of asparagine, and 0.3 ml of DEAE-dextran were added to 40 ml of the enriched medium. Agar (0.5% final concentration) was added to the enriched medium, and underlayers were poured in 35-mm Petri dishes.

After preparation of both bottom and top layers, the plates were examined under the inverted microscope to assure the presence of a good single-cell suspension. The plates were then incubated at 37° in a 7.5% CO\(_2\) humidified atmosphere.

Scoring and Identification of Colonies in Cultures. Cultures were examined with a Zeiss inverted-phase microscope at \( \times 30 \), \( \times 100 \), and \( \times 300 \). Colony counts were made 5, 10, 15, and 20 days after plating. Aggregates of 50 or more cells were considered colonies. Aggregates of less than 50 cells were considered clusters. To more specifically identify colony morphology, colonies were plated with a Pasteur pipet, centrifuged onto a glass slide, and stained with Wright-Giemsa. These slides were compared to sections taken from the original tumor by an experienced pathologist (C. Reichert).

To perform electron microscopy studies, Petri dishes containing neuroblastoma colonies were flooded with glutaraldehyde:paraformaldehyde fixative (9) and allowed to stand for 1 hr at room temperature. After fixation, the upper layer containing the cells was gently removed, postfixed in 1% osmium tetroxide, dehydrated through a series of graded ethanol and propylene oxide, and flat-embedded in Spurr’s medium (20). Sections (1 \( \mu \)m) were cut on a Porter-Blum ultramicrotome, stained with methylene blue-azure II (16), and then examined under the light microscope for orientation and detection of colonies. Ultrathin sections were then obtained, stained with uranyl acetate (24) and lead citrate (15), and examined using a 100S JEOL electron microscope. The original bone marrow tumor cells from which the soft-agar neuroblastoma colony was derived were fixed and processed in a similar manner.

Marker and Karyotype Studies. Culture plates with neuroblastoma colonies were selected on Days 7, 14, 21, and 28. Two ml of Hank’s balanced salt plus 10% fetal calf serum were added to the top of the plates, and the plates were incubated at 37° for 24 hr. The supernatant was decanted and sent for determination of catecholamines by a standard colorimetric technique (8).

Cytogenetic analysis of agar cultures was performed as described by Trent (22). Briefly, cultures were overlaid with 0.1 \( \mu \)M colchicine in 2.5 ml of CMRL 1066 medium. Five hr later, the agar upper layer (containing the tumor colonies) was removed and was centrifuged for 5 min at 150 \( \times \) g. The supernatant was then removed, and the upper layers were exposed to 0.075 M KCl for 25 min at 37°. Following hypotonic treatment, tumor colonies (within residual agar) were centrifuged, the supernatant was removed, and cells were fixed with absolute methanol:glyceraldehyde acetic acid (3:1). Following preparation of air-dried slides, standard Giemsa and G-banding (21) were performed.

RESULTS

Development and Identification of Colonies. Cell doublings were usually observed within 12 hr of plating, and clusters of 8 to 20 cells usually appeared within 3 to 5 days. Colonies (collections of 50 or more cells) appeared 8 to 10 days after plating. Cell lysis generally occurred 35 to 40 days after plating. Cultures were not refed nor was any attempt made to subculture the colonies. The appearance of a typical culture plate on Day 10 is shown in Fig. 1. It is noteworthy that colonies developed at different rates. Colonies were tightly packed, consisting of 50 to several hundred small uniform round cells (about 10 \( \mu \)m in diameter).

The number of colonies which grew from histologically positive bone marrows ranged from 6 to 7,214 per 500,000 nucleated cells plated, yielding a plating efficiency (number of colonies per number of nucleated cells plated) of 0.0012 to 1.44% (median plating efficiency, 0.005%; mean, 0.087%). A linear relationship was obtained between the number of nucleated cells plated and the number of colonies found after 15 days (Chart 1). The percentages of tumor-plating efficiencies in 2 lymph node specimens were 0.0238 and 0.047% versus 0.004 and 0.1404%, respectively, in the bone marrow of the same patients. The plating efficiency of the solid tumor specimen which grew was 0.0076%.

To prove that the colonies growing in culture were indeed composed of neuroblastoma cells and not fibroblasts or granulocytes and macrophages, 4 approaches were used. These included light microscopy, electron microscopy, serial tumor marker determinations, and karyotyping.

Light Microscopy of Colonies. After preparations of slides from the agar plates as described above, the slides were stained with Wright-Giemsa, peroxidase, or nonspecific esterase stains (10). Wright-Giemsa stain demonstrated colonies composed of collections of cells slightly larger than lympho-
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Chart 1. Linear relationship between colony formation and the number of nucleated bone marrow cells plated. Points, mean of 3 dishes; bars, S.E. These are the results of a typical experiment.

Fig. 1. A, typical culture plate demonstrating neuroblastoma colonies growing on Day 10. Note heterogeneity of sizes of clusters and colonies (×30 inverted microscope). B, higher magnification showing details of small cell size (×300 inverted microscope).

Fig. 2. Wright-Giemsa stain of neuroblastoma colony on Day 14. Note colony is composed of a collection of cells, slightly larger than lymphocytes with deeply staining nuclei and an occasional mitosis. ×1,000.

cytos and with deeply staining nuclei (Fig. 2). Mitoses could be seen in a number of the colonies. The histology of these colonies closely resembled tumor from the original biopsy. Peroxidase and nonspecific esterase stains of entire plates revealed an average of 4 positive colonies (range, 0 to 5) per plate. These represented granulocyte-macrophage colonies and usually demonstrated a diffuse starburst pattern characteristic of those colonies.

Electron Microscopy of Colonies. The colony cells examined by electron microscopy showed remarkable morphological similarity to the tumor cells in bone marrow (Fig. 3). The colonies consisted of concentric aggregates of cells, the center of which was composed of mainly necrotic cells with a periphery of morphologically intact cells. Mitotic cells in the colony were also seen. The cells had large nuclei with prominent nucleoli and dispersed chromatin. The cytoplasm contained all the typical ultrastructural features of neuroblastoma, namely, dense-core secretory vesicles, cell processes, and extensions containing microtubules and occasional desmosomes (11, 17). Also present were lipid vacuoles, mitochondria, ribosome clusters, endoplasmic reticulum, and golgi apparatus.

There were 2 differences observed between the bone marrow specimen and the agar colonies: (a) the desmosomal attachments linking the tumor cells were more frequent and extensive in the bone marrow samples than in the agar colonies; and (b) mitotic activity was increased in the agar colonies compared to the tumor cells in the bone marrow. This would be further evidence that colonies were actually being generated and refutes the argument that clumps of tumor from bone marrow are being seeded onto the agar.

Marker Studies. Marker studies were performed on cultures from 3 different patients who had elevated levels of urinary catecholamines. Chart 2 demonstrates that the level of catecholamines in the tissue culture media increased with increasing time in culture as compared to control plates containing growing ovarian cancer colonies.

Karyotype Analysis. Cytogenetic analysis was attempted on 4 tumors grown in soft agar with 3 specimens yielding sufficient mitoses for modal chromosome assessment. Modal chromosome numbers of the 3 tumors studied were derived from direct counts of 300 of the over 2000 mitotic figures observed. All 3 tumors contained near-diploid modal chromosome numbers with apparently minimal chromosome change. One patient displayed a hyperdiploid mode at 60 (with 10% polyploidy); the remaining 2 evidenced hypodiploidy, the first with a bimodal distribution at 43 to 44 and the second with a modal number of...
Fig. 3. A, neuroblastoma colony grown in soft agar showing the tumor cells arranged in a concentric pattern. Necrotic and degenerating cells can be seen in the center. ×2,600. B, cell process of colony cell showing microtubules (thin arrow) and dense core secretory granules (thick arrow). ×39,000. C, Desmosomal junction (arrow) linking the cytoplasm of 2 cells growing in a colony. ×52,000.

Chart 2. Levels of catecholamines in supernatant of neuroblastoma colonies in culture with increasing time in culture (A, □, □). Control plates are ovarian cancer cells growing under same conditions (○).

45. Neither double minute bodies nor homogeneously staining regions were observed in any tumor studied. The finding of near diploid mitoses in direct samples from human neuroblastomas has been reported previously by Brodeur et al. (2). Additionally, although double minute bodies and homogeneously staining regions are common elements of neuroblastoma cell lines (1), direct tumor samples have evidenced double minute bodies or homogeneously staining regions in less than 20% of cases (2). Despite the absence of double minute bodies in these 3 tumors, the presence of stable clones with aneuploid chromosome numbers is suggestive of the malignant origin (rather than origin from normal cellular contaminants) of cells forming the colonies in vitro. Results of detailed chromosome banding analysis will be presented elsewhere.3

Sensitivity and Specificity of Method for Diagnosing Neuroblastoma. There were a total of 76 specimens (71 marrow, 3 lymph node, 2 solid tumors) for which both routine histological examination and soft agar culturing results were obtained. Table 1 details the significant correlation between histological and soft agar assay results (p < 0.001, χ² test corrected for continuity). There were 38 instances in which histology of bone marrow (34 specimens), lymph node (3 specimens), or primary tumor (1 specimen) demonstrated neuroblastoma cells. The agar culture showed colony growth (≥5 colonies/plate) in 30 of these instances (26 bone marrows, 3 lymph node, and 1 solid tumor) (true positives), whereas in 8 instances the soft agar system did not demonstrate colony growth (false negatives).

There were a total of 38 specimens which were histologically negative for neuroblastoma. Thirty of these 38 specimens also showed no growth in the agar system (true negatives). However, 8 histologically negative specimens formed colonies in the soft agar system (median of 5 colonies/plate; range, 5 to 9). The cells growing in these colonies in soft agar were examined in 2 instances by light and electron microscopy and were found to be neuroblasts with features identical to those in Fig. 2 and 3. These 8 histologically negative but soft agar-positive specimens were from 6 different patients. It is possible that the histological examinations of these 8 specimens in the 6 patients were falsely negative since 5 of the 6 patients had

Table 1

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* These 8 specimens were derived from 6 patients. Five of these 6 patient specimens did have positive histologies on prior or subsequent marrow examinations.

Histologically documented evidence of neuroblasts within the bone marrow either prior to (3 patients) or within 2 months after (2 patients) the histologically negative bone marrow specimen. One of the patients with growth of colonies in the soft agar system never had or never developed a histologically positive marrow, and that patient's specimen is considered a definite false positive for the soft agar culture system. Unfortunately, histological studies of the colonies growing in soft agar were not performed on that specimen.

The relationship between histological status of the specimen and the number of colonies growing in culture is demonstrated in Chart 3. It should be noted that of the 38 histologically negative specimens, only 8 formed more than 5 colonies/plate in the soft agar system (an average of 1.8 colonies for all 38 specimens; range, 0 to 9; median, 0.8 colony/plate), whereas for the 38 histologically positive specimens 30 formed more than 5 colonies (an average of 40 colonies; range, 0 to 7314; median, 19 colonies/plate). There exists a highly significant association between the number of colonies per plate and histological status (p < 0.005, Wilcoxon rank-sum test).

Serial Studies and Clinical Correlations. A total of 7 patients with histologically proven bone marrow metastases at the time of diagnosis were studied with serial marrow specimens (Chart 4). There are several interesting findings. Five of the 7 patients (Patients 1, 2, 3, 6, and 7) whose plating efficiency increased to >0.1% died within 4 weeks of that point. Patients 3, 4, 5, and 6 all exhibited a decrease in their plating efficiencies which correlated with regression of their disease (Patients 3, 4, and 6) or a complete clinical response (Patient 5). Patient 4 was lost to follow-up and died of progressive disease 4 months later.

DISCUSSION

These studies have demonstrated that tumor cells from patients with neuroblastoma can form colonies in soft agar. Four lines of investigation provided evidence the colonies growing in the soft agar were composed of cells resembling the neuroblastoma cells growing in the patient. The colonies had light and electron microscopic characteristics of the parent neuroblastoma. The tumor cells in the colonies secreted catecholamines in culture as they did in the patient with levels of catecholamines increasing with time in culture. This phenomenon correlates well with the recently described secretion of tumor markers by other tumors growing in a soft agar system (23). Cytogenetic analysis revealed aneuploid chromosome numbers in the cultured cells which is suggestive of a malignant origin of cells forming the colonies in vitro. The present report is not the first report of growth of neuroblastoma colonies in soft agar. Growth of one of five neuroblastomas in a soft agar system has been reported previously by McAllister and Reed (13), and growth of neuroblastoma from one patient was also reported by Hamburger and Salmon (5).

One recurring question is whether the media used in the soft agar system provided an environment that supported the growth of neuroblastoma colonies. The present report suggests that the media used in our soft agar system would support the growth of neuroblastoma colonies. The media used in our soft agar system included fetal bovine serum, antibiotic/antimycotic solution, and 20% horse serum. The fetal bovine serum and antibiotic/antimycotic solution were obtained from the same supplier as McAllister and Reed (13), and the horse serum was obtained from the same supplier as Hamburger and Salmon (5).

Chart 3. Relationship between histological findings in bone marrow (negative or positive for neuroblastoma) and the number of colonies growing in culture. See text for discussion.

Chart 4. Plot of % plating efficiency [(number of colonies/number of cells plated) x 100] of serial marrow samples using the soft agar system versus time.
agar system is specific for growth of tumor cells. Histochemical stains of slides made from the soft agar revealed that an average of only 4 granulocyte-macrophage colonies were present on each plate (5 × 10^5 nucleated cells plated). More importantly, this blinded study has demonstrated that there is a very good correlation between histological evidence of marrow involvement by neuroblastoma and growth of tumor colonies in this soft agar system. When compared to histological examination of the marrow, the accuracy of the soft agar system for a positive diagnosis of neuroblastoma in the marrow was (true positive/true positive + false negative) = 0.79 (sensitivity) while the accuracy of the soft agar system for finding a marrow free of tumor was also 0.79 (specificity). There were 8 samples from 6 patients in which the soft agar assay grew >5 colonies/plate, but the marrow was histologically negative (called a false-positive soft agar assay). The cells growing in these colonies were examined in 2 instances by light and electron microscopy and were found to be neuroblasts. In 5 of the 6 patients, the marrow had histologically documented neuroblastoma in the bone marrow within 2 months before or after the histologically negative marrow. Thus, in these 5 cases, it is conceivable that growth in the soft agar system was a more sensitive index of the presence of tumor than the histological examination of the bone marrow. Unfortunately, individual colonies from all 6 of the 8 "false-positive" specimens were not examined by light and electron microscopy. This will have to be done in future studies to elucidate the diagnostic capabilities of this cloning system.

The 8 instances where the soft agar assay did not grow tumor from histologically positive marrows (false-negative stem cell assay) are bothersome. Reasons for this failure to grow could be related to recent (within 1 week) chemotherapy (in 3 instances), sampling problems, or technical problems (shipping in the mail for 48 hr). It seems unlikely that mail shipment makes a difference since all bone marrow specimens had ≥90% viable cells when received including the 8 "false-negative" specimens in soft agar. Further studies on the effect of delay in culturing and effects of recent chemotherapy are needed. It is possible that even though tumor cells were seen on histological examination these tumor cells were not able to replicate because of lack of appropriate conditions in the culture system. It is unlikely that there are some patients' tumors that have such a low clonogenic ability that they will never grow in soft agar. The 8 false-negative cultures were from bone marrows from 6 different patients (2 patients had 2 false-negative cultures). The 2 patients who had 2 marrows cultured which were both false negatives in soft agar had had 5 and 2 other bone marrows cultured, respectively, which were all clearly positive for tumor growth.

Most interesting in this study was an apparent relationship between percentage of plating efficiency of the tumor and the clinical course of the disease. Although the number of patients is small, when a patient's tumor plating efficiency increased, this signaled a clinical relapse of disease. This was evident in 5 patients. In these 5 patients, the tumor-plating efficiency became greater than 0.1%. All of these patients died within 1 month of that determination. A decrease in tumor-plating efficiency was consistent with a good response to chemotherapy in 4 patients. It is entirely possible that the clinical response of these patients could have been predicted by measurement of the usual clinical parameters and histological examination of the bone marrow. However, clumps of neuroblastoma in the bone marrow are often difficult to find and identify with certainty. More routine surveillance of patients' marrows using soft agar culture techniques could conceivably detect relapses earlier.

Following plating efficiencies with a soft agar culture system could also provide an excellent parameter for measuring tumor response. Patients whose tumors will not respond could be identified, and early changes to more effective chemotherapy could avoid the continuation of a therapy regimen that is ineffective against the tumor but still toxic to the patient.

The ability to grow neuroblasts from bone marrow specimens in a soft agar system has been documented. Since approximately 70% of children with neuroblastoma have marrow involvement at the time of diagnosis, tumor is accessible for sampling in a majority of the patients. Careful prospective clinical testing of this soft agar technique appears warranted to determine its utility in diagnosis, selecting the most effective drug for an individual patient with neuroblastoma and following the clinical course of a patient with neuroblastoma.

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