Cloning of Human Neuroblastoma Cells in Methylcellulose Culture

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ABSTRACT

An in vitro methylcellulose technique was used in an attempt to culture neuroblastoma cells from 25 bone marrows from eight children with neuroblastoma. Colonies appeared within 5 days in histologically positive bone marrows. Light microscopy, linearity study, and marker study provided evidence for the neuroblastoma origin of the colonies. These colonies could be distinguished from other colonies under the inverted microscope because of its distinct feature. In one case, the characteristic morphology of neuroblastoma was shown in 3 days of culture, while histological evidence is absent. The diagnosis of neuroblastoma was confirmed by subsequent catecholamine determination. All histologically negative specimens formed no colonies, while all positive specimens formed more than three colonies. Potential application of this culturing technique for monitoring of bone marrow involvement and differential diagnosis in children with neuroblastoma is presented.

INTRODUCTION

In spite of the introduction of intensive combination chemotherapy, the prognosis for children with neuroblastoma has improved minimally in recent years. This is possibly related to the fact that most neuroblastoma patients have disseminated disease at diagnosis, and there are few drugs of known effectiveness available for systemic therapy (1). Children with neuroblastoma with bone marrow involvement are classified as having Stage IV or IV-S neuroblastoma, and bone marrow involvement is associated with a poor prognosis.

Recently, an in vitro cloning of neuroblastoma cells using an agar culture system was developed in several laboratories (2-4). This paper describes the growth of neuroblastoma cells in the methylcellulose culture system. Potential application of this culturing technique for monitoring of bone marrow involvement, differential diagnosis, and prognosis in children with neuroblastoma is presented.

MATERIALS AND METHODS

Patients. Twenty-five bone marrow aspirates were performed on 8 children with neuroblastoma or suspected neuroblastoma. At the time of diagnosis, 6 patients had Stage IV disease, and 2 were Stage III, according to the criteria of Evans (5).

Preparation of Cell Suspensions. Bone marrow was aspirated into a syringe containing preservative-free heparin, layered on Ficoll-Hypaque, and centrifuged at 1500 rpm for 25 min. Mononuclear cells were harvested and washed twice in Iscove's modified Dulbecco's medium. The cells were passed through 25-gauge needles until a single-cell suspension was obtained.

Clonal Cell Culture. Methylcellulose culture, a modification of the Chinai Cell Culture. Methylcellulose culture, a modification of the technique described by Iscove (6), was used. Bone marrow mononuclear cells (0.5 to 5 x 10^6) were suspended in Iscove's modified Dulbecco's medium containing 0.96% methylcellulose (Fisher Scientific), 20% fetal cow serum, 5 x 10^{-3} M 2-mercaptoethanol, and 20% phytohemagglutinin-stimulated leukocyte-conditioned medium. The culture mixture was placed in a 35-mm Petri dish, and the plates were examined under an inverted microscope to assure the presence of a good single-cell suspension. Clumps of 2 or more cells were not observed with the present method. The plates were then incubated at 37°C in a 5% CO_2-humidified atmosphere. Cultures were examined with an inverted microscope at x100 and x400. Colony counts were made 3, 7, and 14 days after plating.

Identification of Colonies in Culture. Cell aggregates of more than 50 cells were scored as colonies under the inverted microscope. For identification of neuroblastoma cells, colonies were removed from the dishes with Pasteur pipets and smeared on slides. Colony smears were stained with Wright-Giemsa stains.

Marker Analysis by Using NSE Reactivity. One hundred colonies in methylcellulose culture were removed from the dishes and smeared on slides. Colony smears were stained by use of the avidin-biotin-peroxidase complex in immunoperoxidase techniques as described by Hsu et al.

RESULTS

Neuroblastoma Colonies. Neuroblastoma colonies of <50 cells usually appeared within 2 to 3 days. Neuroblastoma colonies (a collection of more than 50 cells) appeared 3 to 4 days after plating and had maximal colony formation between Days 12 and 16 of culture. Cell degeneration occurred 30 to 40 days after plating. Based on this information, the neuroblastoma colonies were routinely scored on Day 14 of culture. Fig. 1 is an example of a typical neuroblastoma colony on Day 14. Neuroblastoma colonies were compact, tightly packed colonies, containing uniform round, light-brown colored cells with occasional neurite outgrowth. Because of these distinct features, neuroblastoma colonies could be distinguished from other colonies (Fig. 2). Wright-Giemsa staining of the cells from neuroblastoma colonies is shown in Fig. 1. Individual cells revealed the same morphological characteristics as did tumor cells in the original bone marrow. A linear relationship was obtained between the number of cells plated and the number of colonies (Fig. 3).

Marker Studies. All cells from neuroblastoma colonies were NSE positive, although the amounts were variable. On the contrary, all cells from neutrophil, macrophage, and eosinophil colonies were NSE negative.

In one patient, when diagnostic histological and laboratory evidence was absent, the characteristic morphology of neuroblastoma was shown in 3 days of culture. Subsequent examination of the quantitative catecholamine determination and computerized tomography scan confirmed the correct diagnosis of neuroblastoma.

The relationship between histological status of the specimen and the number of neuroblastoma colonies growing in culture is demonstrated in Fig. 4. Sixteen histologically negative specimens showed no growth in the methylcellulose culture system. The number of neuroblastoma colonies which grew from histologically positive bone marrows ranged from 3 to 4,080 per
CLONING OF HUMAN NEUROBLASTOMA CELLS

Fig. 1. A, neuroblastoma colony growing on Day 14. The typical pattern of neurite outgrowth can be seen. B, a portion of a smear from a neuroblastoma colony stained with Wright-Giemsa.

500,000 mononucleated cells plated, yielding a plating efficiency of 0.0006 to 0.8%. There exists a highly significant association between the number of neuroblastoma colonies and histological status of bone marrow involvement.

Hematopoietic Colonies. Histologically negative specimens formed normal numbers of neutrophil, macrophage, eosinophil, and erythroid colonies as compared to the normal control, while no neuroblastoma colonies were formed. The number of hematopoietic colonies which grew from histologically positive bone marrows ranged from 0 to 355 per 500,000 mononucleated cells plated. The neutrophil, macrophage, eosinophil, and erythroid colonies grew side by side with the neuroblastoma colonies.

DISCUSSION

Growth of neuroblastoma colonies in a soft agar system has been reported previously by several investigators (2-4). The results presented in this study demonstrate that human neuroblastoma colonies can be grown in methylcellulose. These colonies appeared 3 to 4 days after harvesting and could be easily distinguished from other colonies under an inverted microscope.

Fig. 2. A, typical neutrophil colony at 14 days grown in methylcellulose; B, macrophage colony; C, eosinophil colony.
cases of neuroblastoma has been well documented. In one case the characteristic morphology of neuroblastoma was shown in 3 days of culture, while histological evidence was absent. The diagnosis of neuroblastoma was confirmed by subsequent time-consuming quantitative catecholamine determination. Murray (8) reported that the neurite outgrowth from clumps of tumor tissue explanted into plasma clot culture readily distinguished neuroblastoma from other tumors of similar histological appearance. Reynolds (9) reported that the most sensitive diagnostic methods were morphology in short-term culture. When the histopathology does not provide a definite diagnosis, methylcellulose clonal cell culture can be helpful in identifying neuroblastoma.

There is a very good correlation between histological evidence of marrow involvement by neuroblastoma and growth of tumor colonies in the methylcellulose culture system. All histologically negative specimens formed no colonies, while all positive specimens formed more than 3 colonies. Detailed examination of 2 bone marrows growing 3 colonies, which at first had been considered to be negative for marrow involvement, revealed a small number of clumps of neuroblastoma. Von Hoff et al. (4) reported that 8 histologically negative specimens formed colonies in the soft agar system, and in those specimens' culture system was a more sensitive index than the histological examination of the bone marrow. The number of patients in this study is so small that further studies are needed to determine if methylcellulose culture might detect the bone marrow involvement when histological evidence is absent. Von Hoff et al. (4) also found that the increased plating efficiency indicated tumor relapse and that the decreased one was consistent with a good response to chemotherapy. All of the 5 patients whose plating efficiency became greater than 0.1% died within 1 mo of that determination. In our study, a relapsed patient, whose tumor-plating efficiency became 0.8%, responded well to chemotherapy, but eventually died 6 mo later.

Most of the investigators used an agar culture system as described by Hamburger and Salmon (10). Experiments performed by Buick et al. (11) and Pavelic et al. (12), who used a methylcellulose monolayer system, revealed insufficient tumor colony formation. Recently, however, Neumann et al. (13) and Cillo et al. (14) reported that they were able to grow sufficient tumor cell colonies in a methylcellulose monolayer system. The agar system may facilitate the colony growth of tumor cells depending on the type of tumor (12). However, the methylcellulose monolayer system might be easier to handle with respect to the plating procedure and might be less time consuming (13). One of the major advantages of this system over the agar system resides precisely in the easy handling of individual colonies (14).

The big difference between our report and those of others is that our culture system is not specific for the growth of tumor cells only. Originally, this culture system is for the growth of hematopoietic stem cells, and by the addition of erythropoietin, various kinds of multipotent stem cells can grow together with neuroblastoma. It is the opinion of the authors that a culture system, which is specific only for the growth of particular tumor cells, may be more sensitive than the methylcellulose system. The agar system may facilitate the colony growth of tumor cells depending on the type of tumor (12). However, the methylcellulose monolayer system might be easier to handle with respect to the plating procedure and might be less time consuming (13).

### Table: Relationship between histological findings in bone marrow and the number of colonies grown in culture.

<table>
<thead>
<tr>
<th>Histology</th>
<th>Number of neuroblastoma colonies grown</th>
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<tbody>
<tr>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>Positive</td>
<td>150</td>
</tr>
<tr>
<td>Negative</td>
<td>50</td>
</tr>
<tr>
<td>Positive</td>
<td>0</td>
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Because of their distinct features. The colonies growing in methylcellulose were composed of cells resembling the neuroblastoma cells in the patient's bone marrow. These colonies, rising from cells of single neuroblastoma, were demonstrated by linear relationship between the number of cells plated and colonies. In the report by Von Hoff et al. (4), extensive uses of histochemical methods, electron microscopic study, marker studies, and cytogenetic analysis were made to identify the malignant origin of the colonies grown in agar. In the present report, immunostained studies with antiserum to NSE provided further evidence for the neuroblastoma origin of the colonies, and neurite outgrowth from the cells in these colonies provided direct evidence for neuroblastoma.

The difficulty in establishing the correct diagnosis in some...
In summary, the ability to grow neuroblasts from bone marrow specimens in a methylcellulose system was documented. This culturing system appeared to be useful for the monitoring of bone marrow involvement, differential diagnosis, and predicting clinical relapse. Additional studies are needed to determine if this culture will be useful in predicting clinical responses to chemotherapy, immunotherapy, and radiation therapy.

REFERENCES

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