Rhabdomyosarcoma Spheroids with Central Proliferation and Differentiation

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Abstract

A novel type of multicellular spheroids was established and characterized with regard to growth behavior, proliferation, and differentiation. The spheroids were grown from clonal rat rhabdomyosarcoma cells using the spinner flask technique. The cell aggregates showed several unique properties that were different from those observed in most of the spheroids investigated to date. These properties include a non-Gompertizian volume growth; the coexistence of undifferentiated mononuclear cells and of differentiated, myotube-like giant cells with numerous nuclei; a relatively homogeneous intraspheroidal distribution of proliferating mononuclear cells with thymidine labeling even in the center of spheroids >1 mm; the absence of necrosis in such large spheroids, and the accumulation of myotube-like cells in the center of these spheroids instead. There was a decrease in the overall proliferative activity of the mononuclear cells with increasing proportion of giant cells in the rhabdomyosarcoma spheroids.

Introduction

Multicellular tumor spheroids are well established in in vitro systems for studies on the interrelationship among proliferation, metabolic state, and viability of tumor cells under well-defined conditions (1-3). Regional gradients of nutrients and toxic waste products have been demonstrated in these cell aggregates and were correlated with the growth pattern, the clonogenity of cells, and the onset of necrosis (4, 5). Numerous similarities between tumors in vivo and spheroids have been documented (6). In this report, we present data on the growth kinetics, proliferation, and morphology of novel spheroids derived from rhabdomyosarcoma clone C cells (7, 8). These rhabdomyosarcoma spheroids are different from all other spheroids reported to date with regard to several biological properties, such as the emergence of necrosis or the coexistence of morphologically undifferentiated cells and myotube-like giant cells. Clone C spheroids may thus enlarge the spectrum of spheroid models that can be specifically exploited for systematic studies on cellular viability, proliferation, and differentiation in three-dimensional tissue-like structures, e.g., by measuring gradients of oxygen and substrates in these spheroids.

Materials and Methods

Clone C cells were derived from the rat rhabdomyosarcoma cell line BA-HAN-1 as previously described by Gerharz et al. and Gabbert et al. (7, 8). The cells were subcultured by standardized techniques (see, e.g., Ref. 9) using Eagle’s basal medium (Sigma, Deisenhofen, Germany) with the addition of D-glucose to a final content of 4.5 g/liter, of 0.84 g/liter sodium bicarbonate, 10 mmol 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10% (v/v) fetal calf serum (Flow, Meckenheim, Germany), 10,000 IU/liter penicillin, and 10 mg/liter streptomycin (Flow).

Spheroid growth was initiated in microbiological Petri dishes and was maintained in spinner cultures as described earlier (2, 3, 9). The culture medium was identical with that used for monolayers. Mean spheroid volume as a function of time in spinner culture was determined with a standardized protocol commonly used in spheroid suspension cultures (9). The experimental data obtained were approximated by the Gompertz function (10, 11) using a nonlinear least square fitting procedure modified from Marquardt (12).

Spheroids of all sizes were fixed in Bouin’s solution, wax embedded, and serially sectioned into 5-μm-thick slices using a microtome (Reichert-Jung, Nussloch, Germany). The sections were stained with hematoxylin and eosin. Central sections were obtained in the central section. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. Supported by the Deutsche Forschungsgemeinschaft (Mu 576/2-4) and is part of the thesis of U. K.

Received 9/3/91; accepted 11/15/91.

1 The abbreviation used is: TLI, [3H]thymidine labeling index.

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Results and Discussion

Unlike most of the spheroids studied earlier (2, 3, 15), spheroids of clone C cells showed a volume growth kinetic that deviated systematically from the Gompertzian type. Respective experimental values are shown in Fig. 1, including a Gompertzian curve as a best fit to these data. Volume growth stagnated at day 26 with a final volume (mean ± SD) of (5.52 ± 2.51) x 10^{-4} \text{cm}^3 or a diameter (mean ± SD) of 996.7 ± 150 \mu m. Although spheroid growth did not follow a simple Gompertzian kinetic, the initial volume growth rate and the maximum volume at growth arrest were similar to those reported for other spheroid systems (9, 15).

Spheroids of clone C consisted of loosely apposed mononuclear cells and myotube-like multinuclear giant cells, which is illustrated by Fig. 2. The distribution of nuclei within the giant cells was nonhomogeneous but differed from that in terminally differentiated muscle cells. Fig. 2a demonstrates one of the most striking findings with regard to the structure of these spheroids: although sizes of more than 1 mm were reached, the central portion of clone C spheroids contained no necrosis but showed an accumulation of structurally intact giant cells. This unique spatial pattern of differentiated and undifferentiated cells was highly reproducible in various spheroid populations investigated up to now.

Such behavior is similar to that of developing tissues that are often growing as rapidly as many neoplastic tissues yet rarely show necrosis. It is possible that a "switch" to the differentiated state avoids necrosis. The underlying mechanisms may be systematically studied now using these rhabdomyosarcoma spheroids. Another factor contributing to the absence of necrosis may be a better transport of nutrients and wastes through the spheroids as a consequence of loose cellular packing (Fig. 2a). Future investigations of the metabolic milieu in these aggregates have to test this hypothesis. Finally, it has to be taken into account that...
Thin myofilaments (6-8 nm in diameter) were intermingled with thick myofilaments (12-15 nm in diameter) were randomly distributed throughout the cytoplasm of multinuclear cells. The multinuclear cells showed bundles of intermediate filaments but only modest amounts of rough endoplasmic reticulum (see Fig. 2, b and c). Thick myofilaments (12-15 nm in diameter) were randomly distributed throughout the cytoplasm of multinuclear cells. Thin myofilaments (6-8 nm in diameter) were intermingled with these thick myofilaments but visualized to a lesser degree. Myofilibrils with a typical sarcomeric organization and basement membranes around the multinuclear cells were missing. Collagen fibrils were not observed in these spheroids.

Ultrastructural analysis revealed morphological features of rhabdomyogenic differentiation such as thick myofilaments in giant cells but not in mononuclear cells. The multinuclear cells showed bundles of intermediate filaments but only modest amounts of rough endoplasmic reticulum (see Fig. 2, b and c). Thick myofilaments (12-15 nm in diameter) were randomly distributed throughout the cytoplasm of multinuclear cells. Thin myofilaments (6-8 nm in diameter) were intermingled with these thick myofilaments but visualized to a lesser degree. Myofilibrils with a typical sarcomeric organization and basement membranes around the multinuclear cells were missing. Collagen fibrils were not observed in these spheroids.

There was only a slight, insignificant decrease in the labeling index TLI from superficial to central cell areas as observed in autoradiographs of the spheroids investigated. Labeled nuclei could not be detected in myotube-like giant cells. Regional and overall TLIs are summarized in Table 1. TLI values in these spheroids were 5-10 times less than those observed in other aggregate types (4). Also, the spatial distribution of labeled cells in clone C spheroids was much more homogeneous than in all other spheroids investigated to date. Typically, spheroids exhibit a pronounced proliferative gradient with intensive [3H]thymidine labeling in superficial cell layers and literally no labeling in inner, still viable cell areas (4, 17). In contrast, labeled mononuclear cells were found in the center of clone C spheroids even at sizes of 1 mm. These labeled cells were often located close to differentiated myotube-like cells that never showed any labeling. This unique distribution of proliferative cells in clone C spheroids may explain why the volume growth rate of these aggregates is not substantially lower than that of many other spheroid types despite different local labeling indices. Obviously, the relatively low TLI in clone C spheroids is compensated for by the occurrence of proliferating cells in all spheroid parts, particularly in central regions. Whereas proliferation in superficial cell layers is associated with the loss of a significant proportion of descendant cells that are shed into the culture medium, this mechanism may play a minor role during cell division in interior regions of the spheroids. One possible explanation of the relatively homogeneous distribution of proliferating cells in these spheroids is an increased ability of mononuclear cells to migrate. This enhanced migration may be a direct interaction between differentiated and undifferentiated cells. One possible explanation for this may be a decreased cell-cell interaction among mononuclear cells upon an increased “dilution” by intermingled giant cells, if one assumes autocrine and paracrine growth stimulation to be important for mononuclear cells. Another interpretation of these data may be a direct interaction between differentiated and undifferentiated cells, e.g., by growth-inhibiting factors released from the myotube-like cells. This aspect of the biological behavior of clone C spheroids has to be clarified in the near future.

**Acknowledgments**

We would like to thank T. Horn, K. Molter, and V. Pohl for their excellent technical assistance and Dr. O. Thews for helpful discussion and computer-based calculations.

**References**


**Table 1 Thymidine labeling index of clone C spheroids as percentage (mean ± SD)**

<table>
<thead>
<tr>
<th>Region evaluated</th>
<th>TLI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>6.1 ± 2.4</td>
</tr>
<tr>
<td>1</td>
<td>7.4 ± 3.2</td>
</tr>
<tr>
<td>2</td>
<td>4.9 ± 3.1</td>
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Fig. 3. Overall TLI in clonal rhabdomyosarcoma spheroids as a function of the relative area of mononuclear cells, i.e., fraction of mononuclear cell area with regard to unit area. n = 10, r = 0.745, P < 0.05.
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