Cultivation of Retinoblastoma Cells: Correlation Between In Vitro Growth Pattern and Histopathology

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Abstract

Purpose: To investigate the correlation between floating versus adherent growth pattern of cultivated retinoblastoma (RB) cells from three patients with RB and their histopathologic features.

Methods: RB cells from three Iranian patients (MM, NR, and MS) were cultivated in Dulbecco’s modified eagle medium (DMEM) supplemented with 15% fetal bovine serum (FBS) for four weeks in each passage. Fresh medium was added on a weekly basis and immunocytochemistry for Synaptophysin was performed. All experiments were performed in duplicate. Growth pattern of the cultivated RB cells was studied during the three consecutive passages and compared among three cases in the light of histopathologic data.

Results: Cultivated RB cells from MM and NR demonstrated an adherent growth pattern in the 2nd week and the pattern was enhanced by the 4th week. The RB tumorspheres adhered to the bottom of the flask while surrounded by fibroblasts. Histopathologic diagnosis in MM and NR was a well-differentiated RB without optic nerve involvement. Such an adherent growth was not observed in cultivated RB cells from MS, in which the histopathologic analysis revealed a poorly-differentiated RB with optic nerve intrusion and prominent choroidal invasion.

Conclusion: The adherent growth pattern of cultivated RB cells might be associated with tumor differentiation and the lack of optic nerve involvement in histopathology.

Keywords: Adherent Growth; Choroidal Invasion; Histopathology; Optic Nerve Intrusion; Retinoblastoma

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INTRODUCTION

Retinoblastoma (RB) is a common intraocular tumor comprising 2-4% of malignancies in children. Newly diagnosed RB is estimated to be 9000 cases annually, and dreadfully, the mortality rate is as high as 70% in low and middle income countries.[1] In Iran, the mortality rate of RB has been reported to be 10-20% higher than developed countries.[2,3]

Retinoblastoma is further classified as well- or poorly differentiated carcinoma. Well-differentiated RB demonstrates cellular features similar to normal retinal cells. Fleurettes and highly organized Flexner-Wintersteiner rosettes with bland nuclei are structures composed of retinoblastoma cells with
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The study was approved by the Ethics Committee of the Ophthalmic Research Center, Shahid Beheshti University in Tehran, Iran, and informed consent was obtained from the patients’ parents after explaining the purpose of study.

The lesional tissue was dissociated by pipetting and digested with 0.25% trypsin in calcium- and magnesium-free phosphate buffered saline. Then, the digested tissue was transmitted into a 15 ml-falcon and centrifuged. The cell pellet was transferred to T25 flask which had been pre-coated with 20% FBS and contained 6 ml DMEM-F12 media. The cells were incubated at 37 degrees centigrade (37°C) in a 95% air-5% CO2 humidified atmosphere. Then every week, one ml of the media containing 15% FBS was added to the flask. RB cells in our series were passaged 3 times every 4 weeks and cultivated cells were examined and photographed daily by using an inverted microscope (Olympus IX71, Japan) equipped with a digital camera. Moreover, the growth curve of cultivated RB cells was determined and the corresponding doubling time was calculated from the exponential growth phase of the growth curve.

Immunocytochemistry for Synaptophysin, as a valuable marker for the detection of cells of neural origin,[14] was performed to detect RB cells. Briefly, RB cells in the 2nd passage were cultured in 24-well plates and fixed in −10°C methanol for 10 minutes. The cells were permeabilized with TritonX-100 (0.25%) and blocked in 1% bovine serum albumin (BSA) in 1X phosphate buffered saline (PBS) for one hour at room temperature. The RB cells were incubated with rabbit anti-human Synaptophysin antibody (1:200; DAKO, Copenhagen, Denmark) for 24 hours at 4°C. Fluorescein isothiocyanate (FITC)-conjugated antibodies (goat anti-rabbit IgG, diluted 1:400; Santa Cruz, Carlsbad, CA) were used for 45 minutes to detect the immunoreactivity of the cultivated RB cells to the primary antibody. After washing gently, the slides were incubated with 4,6-diamidino-2-phenyindole dihydrochloride (DAPI; 1.5 mg/ml; Santa Cruz) for 10 minutes to stain nuclear DNA. The immunoreactive cells were detected by using the inverted microscope equipped with a 460 nm filter for DAPI and a 520 nm filter for FITC-conjugated antibodies. Meanwhile, the growth pattern of cultivated RB cells was evaluated.

RESULTS

Three patients (MM, NR, and MS) with clinical diagnosis of a unilateral sporadic RB in advanced stages were included. No family history of RB was found and the parents did not have a consanguineous marriage. Case number 1 (MM) was an 18-month-old girl with an abnormal light reflex in the right eye. This eye was diagnosed to have RB in the examination under anesthesia and due to its dimension, planned to be enucleated with sufficient excision of the optic nerve (at least one centimeter). Histopathological examination of
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the enucleated eye revealed a well-differentiated RB with Flexner-Wintersteiner rosettes [Figure 1]. Vitreous and anterior chamber seeding of the RB cells was minimal, optic nerve invasion was negative and choroidal expansion was limited.

Case number 2 (NR) was a 7-month-old girl with leukocoria of her left eye and an 8 × 15 mm² white retinal mass observed in funduscopic examination. Histological examination disclosed a well-differentiated RB occupying the entire vitreous cavity and typical features of Flexner-Wintersteiner rosettes. Retrolaminar optic nerve was tumor-free and a minimal choroidal infiltration was observed. Minimal vitreous and anterior chamber seeding of the RB cells was present.

Case number 3 (MS), a 24-month-old girl was presented with strabismus and leukocoria of the left eye. In ophthalmologic examination performed in the operating room, a white-gray, large mass was detected at the posterior pole. Vitreal haziness was evident but anterior segment had not been invaded grossly. The involved eye was enucleated in the same way as described for the previous cases. Histological examination showed a high grade, poorly-differentiated retinoblastoma with an overwhelming marked seeding of the vitreous and anterior chamber angle, large-scale retrolaminar invasion and prominent choroidal involvement. The surgical margin of the recovered optic nerve was tumor free. The Azzopardi phenomenon was a remarkable feature in this case [Figure 2]; however, it was not observed in the other two cases (NR and MM).

In the cultivation of RB cells from MM and NR in all passages, a biphasic population of cultivated cells was observed during the first week, consisting of retinoblastoma tumorspheres and a single-cell suspension over the feeder fibroblastic layer. The feeder layer was adhered to the bottom of the flask. In consecutive evaluation of the plates, the remarkable finding was an early adherence of the RB tumorspheres to the bottom of the flask while surrounded by fibroblastic cells and were immune reactive for Synaptophysin. Over the time, the adherence of tumorspheres to the bottom of flask was intensified over the 4-week period. Such an adherent growth of RB cells was not observed in the cultivated RB cells recovered from MS who possessed a poorly differentiated malignancy [Figure 3]. Furthermore, cell population doubling times calculated from the exponential growth phase of the growth curves in our series ranged from 1 to 2 days [Figure 4]. Patients’ data, histopathological findings and their corresponding culture results are shown in Table 1.

DISCUSSION

Our study demonstrated for the first time to the best of our knowledge, a correlation between growth pattern of cultivated RB cells and corresponding histopathological features in three RB patients. Well differentiated RB, with minimal restricted vitreous seeding and no choroidal and optic nerve violation, disclosed an adherent growth pattern in vitro. However, in poorly differentiated RB, with widespread vitreal seeding and massive intrusion of the optic nerve and choroidal layer, a floating pattern of growth was detected in cultivated RB cells. Our results with respect to the well differentiated RB

**Figure 1.** (a) Well-differentiated RB in case #1 with a mixture of Flexner-Wintersteiner and Homer-Wright rosettes (hematoxylin and eosin stain, magnification ×200). (b) Represented Flexner-Wintersteiner rosettes (arrows) (hematoxylin and eosin stain, magnification ×400). (c) Note the presence of minimal choroidal involvement beneath the retinal pigment epithelium (arrow) (hematoxylin and eosin stain, magnification ×400). RB, retinoblastoma.

**Figure 2.** Representative histologic sections in case #3 with marked choroidal involvement (a, hematoxylin and eosin stain, magnification ×200), Azzopardi effect as basophilic DNA deposition in the wall of venules and capillaries (b, hematoxylin and eosin stain, magnification ×400), and retrolaminar optic nerve intrusion (c and d, hematoxylin and eosin stain, magnification ×100 and ×200, respectively). RB, retinoblastoma.
cases were consistent with the SNUOT-Rb1 cell line in which the primary tumor was a well-differentiated RB presented with small Flexner-Wintersteiner rosettes, some intravitreal seeding and no choroidal and optic nerve invasion in a Korean child. SNUOT-Rb1 was previously demonstrated as a different cell line from Y79 or WERI-Rb1, and with unique characteristics of rapid and adherent mono-layered growth in vitro. The growth rate of cultivated RB cells in our series was between those of SNUOT-Rb1 and Y79.

The floating growth pattern of the poorly differentiated RB case (MS) was compatible with the features found in WERI-Rb1 and Y79 cell lines. The WERI-Rb1 cell line was derived from a 1-year-old girl with sporadic retinoblastoma. The Y79 cell line was derived from a 2-year-old girl with hereditary retinoblastoma. According to the pre-existing reports, the degree of tumor differentiation and presence or absence of choroidal and optic nerve invasion in the primary tumor was not specified. The primary culture of both WERI-Rb1 and Y79 cell lines constructed a suspension of adherent (feeder fibroblasts) and nonadherent cells. The nonadherent cells were selected for the cell line. None of the cell lines disclosed adhesion of the nonadherent cells with time.

Anatomically, cells possess highly specified intercellular proteins, which function as an anchorage-like network and maintain the cells together. It is believed that the linkage proteins are distorted in cancerous cells. Therefore, they are prone to invade nearby tissues and

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**Figure 3.** Representative cultivated RB cells in MM, NR, and MS cases. (a, b) presence of floating RB tumorspheres (arrows) overlying fibroblastic cells (asterisks) in the 1st week of 2nd passage in MM case; (c) adhesion of RB tumorsphere (T) to the bottom of the flask while surrounded by fibroblastic cells in MM case in the third week of 2nd passage; (d) markedly adherent RB cells to the bottom of the flask together with rosette formation (R) in the 4th week of 2nd passage in MM case; (e, f) floating RB tumorspheres (asterisks) in the 1st week (e) and the 4th week (f) of 2nd passage in MS case; (g-i) Adherent tumorsphere (asterisk) surrounded by fibroblastic cells in the 2nd week of 2nd passage in the NR case. Cultivated RB cells (g) from the 3rd passage that were stained positively for the fluorescein isothiocyanate (FITC)-conjugated synaptophysine antibody (green) (h). Note merged image (FITC-labeled synaptophysine and DAPI); the arrow shows a nucleous of a fibroblast (i) and DAPI-stained RB and fibroblastic cells nuclei (blue) (magnification 400×). RB, retinoblastoma.

**Figure 4.** Representative growth curve of cultivated RB cells in case #3. Growth curve of cultivated RB cells were plotted and demonstrating cell population doubling time of 1.1 days when calculated from the exponential growth phase of the growth curve. RB, retinoblastoma.
subsequently metastasize. However, this feature is not at the same level among different cancers.\(^6\) The floating pattern of growth in our study (MS) might confirm a tremendous failure of the intercellular linkage proteins in keeping the cells together and justify the worse prognosis of the disease. In addition, we detected a massive expansion of the tumoral cells in the vitreous cavity, choroid and optic nerve in the above case, which is identical to its pattern of growth \textit{in vitro}.

The disease course in MM, NR and Korean child represented a full-blown well-differentiated cancer. No violation of optic nerve or choroid occurred. Moreover, at the microcellular level, the features of differentiation including Flexner-Wintersteiner rosettes and Fleurettes were demonstrated. More importantly, even, the adherent pattern of growth was a common characteristic among them. This finding suggests an association with the clinical presentation.

Our finding supports the hypothesis that the well-differentiated cancerous cells would somehow conserve the common features with normal cells including the intercellular linkage proteins. Therefore, they would stay together for a longer period of time and have lesser tendency to spread in the body as well as in the cultivation plate. That is why we observed an adherent pattern of growth (MM and NR) \textit{in vitro} contrary to our poorly-differentiated case (MS).

The function of cadherin family was highlighted in some of the studies. A study, conducted by Laurie et al\(^6\) on the mouse retinoblastoma cell lines, detected a significantly low levels of N-cadherin and cadherin-11 expression in cancer cells, which promote the vitreal seeding and ocular invasion of retinoblastoma before metastasis. Interestingly, these mechanisms are similar to human retinoblastoma counterparts. In our study, we did not check the expression of cadherin proteins in the aforementioned three cases. Further studies should be conducted to investigate the expression of cadherin proteins in well- versus poorly-differentiated RBs.

We evaluated the cultivated RB cells from three Iranian patients. We did not investigate a cell line originated from Iranian RB tumor. Establishing a cell line from well differentiated Iranian RB cases is in process. In this study, we attempted to find a significant correlation between the pattern of growth of RB cells \textit{in vitro} and the behavior of retinoblastic tumoral cells \textit{in vivo}. Association between clinical and histopathologic characteristics was aimed but molecular assay was not considered to be accomplished concurrently.

All three cases in our study (MM, NR and MS) as well as the SNUOT-RB1\(^{10}\) and WERI-RB1\(^{9}\) harbored sporadic RB, while the Y79\(^{8}\) was originated from a patient with hereditary RB. However, the adherent growth pattern of cultivated RB cells was observed in MM, NR, and SNUOT-RB1 but not in MS, WERI-RB1, and Y79. Although sporadic versus hereditary nature of RB

### Table 1. Demographic data, histopathologic findings and growth pattern of cultivated RB cells in three Iranian patients

| Patients | Demographic data | Histopathologic findings | Growth pattern |
|----------|------------------|--------------------------|----------------|
|          | Sex | Age (Months) | Inheritance | Differentiation | Flexner-Wintersteiner Rosettes | Fleurettes | Azzopardi effect | Optic nerve invasion* | Choroidal invasion | Vitreous seeding |
| MM       | F   | 18           | NA          | −             | +++                       | +           | +          | +               | +++            | −             | −             |
| NA       | F   | 7            | −           | +             | −                        | −           | −          | +               | −              | +             | −             |
| MS       | F   | 24           | −           | +             | −                        | −           | −          | +               | +              | −             | −             |

**Inheritance:** − means sporadic; Differentiation: + means poorly differentiated, +++ means well differentiated; Azzopardi effect: + means present, − means not present; Vitreous seeding: + means present, − means not present.
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does not seem to play a role in adherent versus floating growth pattern of the cultivated RB cells, further large scale investigations are necessary to prove this theory.

Azzopardi effect is the DNA deposition in the wall of venules and capillaries in areas with large-scale tumor necrosis due to liberation of nucleic acids from degenerating neoplastic cells. This phenomenon was observed in miscellaneous aggressive malignancies such as pulmonary small cell tumor, large cell lymphoma, and Merkel cell carcinoma. Massive cellular turnover in high grade malignancies and consequent mass destruction of tumoral cells and DNA liberation would surpass its clearance and accumulate around the vessels. [16-18] In our study, we observed the Azzopardi phenomenon only in our poorly-differentiated RB case (MS). This finding was not previously reported in the various RB cell lines. Further studies are needed to reveal whether such an observation has been an incidental finding in our case (MS) or might indicate a correlation with the growth pattern of cultivated RB cells.

A unique characteristic of growing in adherence was reported in two Iranian (Middle Eastern) patients (MM, NR) as well as in the Asian cell line (SNUOT-RB1). [10] This was not reported in the cell lines that were introduced by the Western researchers (WERI-RB1 and Y79) and was not observed in one Iranian (Middle Eastern) patient (MS). Whether the genetic and ethnic differences might play a role in differences observed between Western and Eastern RB cases, necessitates further investigations.

In summary, the results of our small case series suggest that the adherent versus floating growth pattern of cultivated RB cells might be correlated with the degree of differentiation of RB. Well-differentiated RB tumors with restricted invasion to ocular structures such as optic nerve and choroid demonstrate an adherent growth in vitro. However, a floating pattern is observed in cultivated RB cells from a poorly differentiated RB which has massively violated the choroid and the optic nerve.

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Conflicts of Interest
There are no conflicts of interest.