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Three-Dimensional Growth and Differentiation of Ovarian Tumor Cell Line in High Aspect Rotating-Wall Vessel: Morphologic and Embryologic Considerations

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Abstract Cancer of the ovary is the leading cause of death from gynecologic malignancy. To understand better these aggressive tumors, the development of in vitro models to study human ovarian cancer is critical However, the establishment of long-term cell lines has been difficult, due to the generalized poor survival of patient tumor cells grown in primary culture. Satisfactory culture systems for ovarian tumor cells have therefore been limited To study cellular interactions involved in the growth and differentiation of these tumors, a cell line was established from a mixed mullerian tumor of the ovary. This cell line, designated LN1, was cultured on microcarrier beads in the high aspect rotating-wall vessel. The tumor cells grown in this vessel readily proliferated without a requirement for cocultivation with a supportive cell layer Evaluation of cellular morphology by phase contrast light microscopy and scanning electron microscopy revealed the presence of three-dimensional multicellular aggregates consisting of multiple cell-coated beads bridged together, as well as scattered aggregates of LN1 cells proliferating as spheroids free in suspension In contrast to conventional culture systems, culture in the high aspect rotating-wall vessel facilitated the generation of multiple cell types that could be recovered These results illustrate the ability of this culture system to provide the biological conditions necessary for pluripotent cell growth.

Key words: heterologous mixed mullerian tumor, ovarian tumor model, pluripotent cell

Mixed mullerian tumors of the ovary account for less than 1% of all ovarian cancer [Geisinger et al., 1987]. These highly aggressive tumors are composed of both epithelial and mesenchymal elements, and are nearly identical in composition to the more commonly encountered mixed mullerian tumor of the uterus. Similar to their uterine counterpart, ovarian mixed müllerian tumors may exhibit a homologous phenotype, in which sarcomatous elements common to mullerian duct derivation are expressed, such as leiomyosarcoma. In contrast, these tumors may be heterologous and contain malignant cell populations extrinsic to müllerian duct origin that may include chondrosarcoma, liposarcoma, osteosarcoma, and rhabdomyosarcoma [Ober, 1959].

The histogenesis of mixed mullerian tumors is controversial and numerous theories have been postulated to account for the simultaneous development of carcinomatous and sarcomatous components. Pfannenstiel was the first to postulate that the variety of cellular components present in mixed tumors of the female reproductive tract may result from metaplasia of pluripotent tissue [Pfannenstiel, 1892]. Subsequently, in the early 1900s, Wilms suggested that these unusual malignancies may derive from primitive Wolffian duct [Wilms, 1900]. It was later hypothesized that embryonic stem cell rests from undifferentiated müllerian tissue may give rise to the multiple malignant cell types present in mixed mullerian tumors [Hill and Miller, 1951]. Additional theories proposed to explain the genesis of these unusual tumors include the collision theory, the compositional theory, and the combination theory [Meyer, 1930]. The collision tumor is postulated to arise from two independent malignant cell populations, carcinomatous and sarcomatous, which are derived from separate primary sites. In contrast, the compositional tumor theory suggests that carcinomatous cells become associated with reactive atypical, but benign, stromal elements. Finally,

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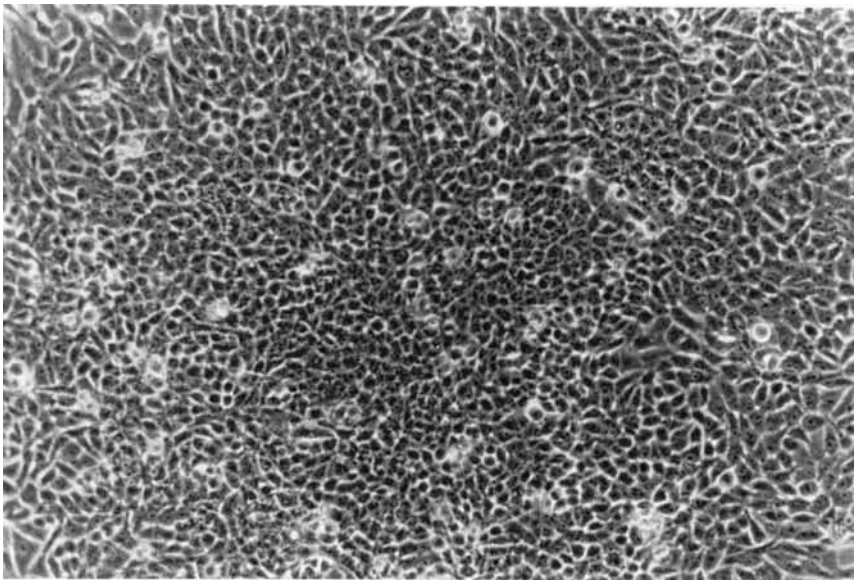

Fig. 1. Morphologic appearance of LN1 cells cultured as a monolayer in vitro (phase contrast ×100) Note the cobblestone-like pattern of growth with distinctive boundaries between cells

the malignant cell populations present in the combination tumor are believed to originate from a single common stem cell.

Over the past decade, the majority of experimental evidence has supported the combination theory for the development of mixed müllerian tumors. Most of these studies evaluated mixed müllerian tumors of the uterus, in which malignant epithelial and mesenchymal cell types are postulated to arise from multipotential endometrial stromal cells [Ishiwata et al., 1981]. Furthermore, it has recently been shown that cell lines derived from heterologous mixed müllerian tumors of the uterus may co-express epithelial and mesenchymal antigens [Emoto et al., 1992]. Few reports have described cell types originating from mixed müllerian tumors of the ovary, although it has been suggested that a common stem cell precursor present in pluripotent ovarian surface mesothelium may give rise to both the carcinomatous and sarcomatous cell populations present in this type of malignancy [Dehner et al., 1971].

We have established the LN1 cell line, which is derived from a heterologous mixed müllerian tumor of the ovary [Becker et al., 1992]. In the present report, we describe the growth characteristics of LN1 cultured in the high aspect rotating-wall vessel (HARV). Previous work has demonstrated the ability of rotating wall vessels (RWVs) to facilitate the differentiation and three-dimensional organized growth of HT29 and HT29KM human colon carcinoma cells and T24 human bladder carcinoma cells [Goodwin et al., 1992; Prewett et al., in press]. The HARV model of LN1 culture affords a novel in vitro system to explore fully the growth potential and capacity for differentiation that these pluripotent tumor cells may exhibit in vivo.

CHARACTERISTICS OF LN1 OVARIAN TUMOR CELL LINE

The LN1 cell line was derived from a metastatic lesion of a patient with mixed müllerian tumor of the ovary. The original tumor was composed predominantly of a poorly differentiated mesenchymal element, with a smaller epithelial component. Foci of leiomyosarcoma and chondrosarcoma were present, and the tumor was classified as a heterologous type of mixed müllerian tumor. The epithelial cells exhibited strong expression of low molecular weight keratin and were negative for other markers including carcinoembryonic antigen. Mesenchymal components of the tumor were highly positive for vimentin; intracellular and extracellular hyaline globules were also noted.

LN1 cells cultured as a monolayer under traditional in vitro conditions exhibit an epithelial-like morphology upon reaching confluency. (Fig. 1). The cells grow as a solid sheet in a cobblestone pattern with distinct intercellular boundaries. These cells are also positive for both low

molecular weight cytokeratin and vimentin, as well as epithelial membrane antigen.

THREE-DIMENSIONAL CELL CULTURE

To determine the three-dimensional growth characteristics and the potential for differentiation of these ovarian tumor cells, LN1 cells from passage 36 were seeded into the HARV on Cytodex-3 microcarrier beads [Prewett et al., 1993]. Cell growth monitored by scanning electron microscopy over 32 days of culture demonstrated the formation of three-dimensional cellular aggregates. These tissue-like aggregates typically achieved maximum diameters of 0.3–0.4 cm (Fig. 2a). It was noted that upon achieving this diameter, the aggregates frequently would break away from the carrier bead and continue to proliferate in the absence of the bead. An example of this type of cellular aggregate is shown in Figure 2b. Thus, the three-dimensional masses of ovarian tumor cells grown in HARV culture were composed of both multiple cell coated beads bridged together, as well as cellular aggregates proliferating free in suspension. This pattern of growth is in contrast to that exhibited by the majority of other cells cultured in RWVs, since most cells grown under these conditions continue to proliferate while attached to the bead, often reaching diameters of 1 cm or greater [Goodwin et al., 1992; Prewett et al., in press]. The tendency for LN1 cells to break away from the bead upon reaching a particular size or cellular density may be a characteristic reflective of the metastatic origin of these cells. Alternatively, this property may be associated with the multiple cell phenotypes present within individual aggregates.

MULTIPLE CELL TYPES GENERATED IN HARV CULTURE

In addition to the striking three-dimensional cellular organization that developed during HARV culture, LN1 cells grown under these conditions generated multiple cell types. Scanning electron microscopy demonstrated the distinctive variation in cellular morphology that occurred during culture in the HARV; diverse populations were evident, with an extensive array of microvilli extending from the surface of the three-dimensional aggregates of cells (Fig. 3). The complexity of these cell populations was further emphasized by conventional in vitro culture of cells grown in the HARV. Figure 4 illustrates a field of view showing multiple cell types

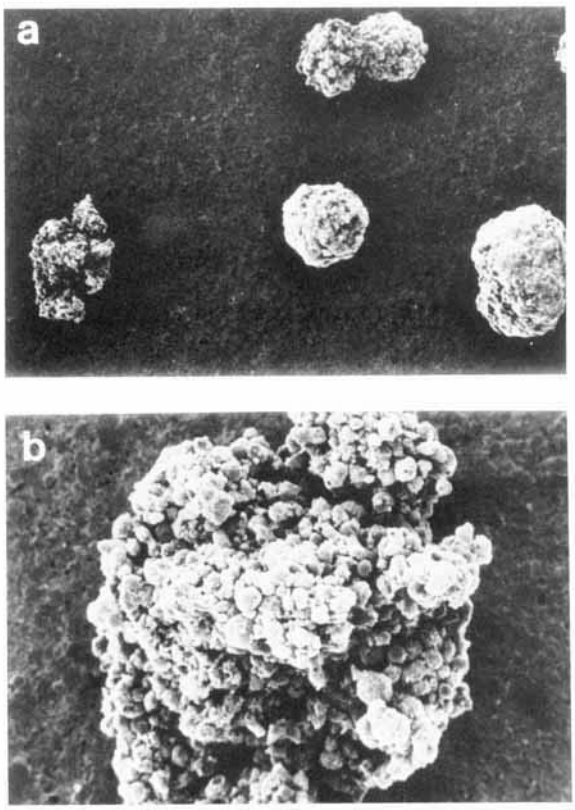

Fig. 2. Scanning electron micrograph of LN1 cells cultured on Cytodex carrier beads in the HARV **a:** Multicellular aggregates composed of cell-coated beads reached diameters of 0.3–0.4 cm (×75) **b:** Aggregate of LN1 cells that has broken away from the carrier bead and continued to proliferate free in suspension (×350)

including elongated spindle-shaped cells and sarcomatous cells exhibiting lipid droplet accumulation (lower right) and a nest of epithelioid cells showing cohesive cobblestone-like growth (upper right). Isolated foci of cells showing polarized accumulation of osteoid material were also observed (Fig. 5), suggesting the regeneration of the chondrosarcoma element that was present in the original tumor specimen. These isolated cell populations were no longer evident following passage of the cells in vitro. Upon reaching confluency, the monolayer showed re-expression of the homogeneous epithelial-like cobblestone pattern of growth.

EMBRYOLOGIC CONSIDERATIONS

These findings have a fundamental impact on the combination theory of mixed müllerian tumor development. The ability of the LN1 cell line to differentiate into diverse subpopulations of cells provides direct evidence in support of this theory for the generation of mixed mülle-

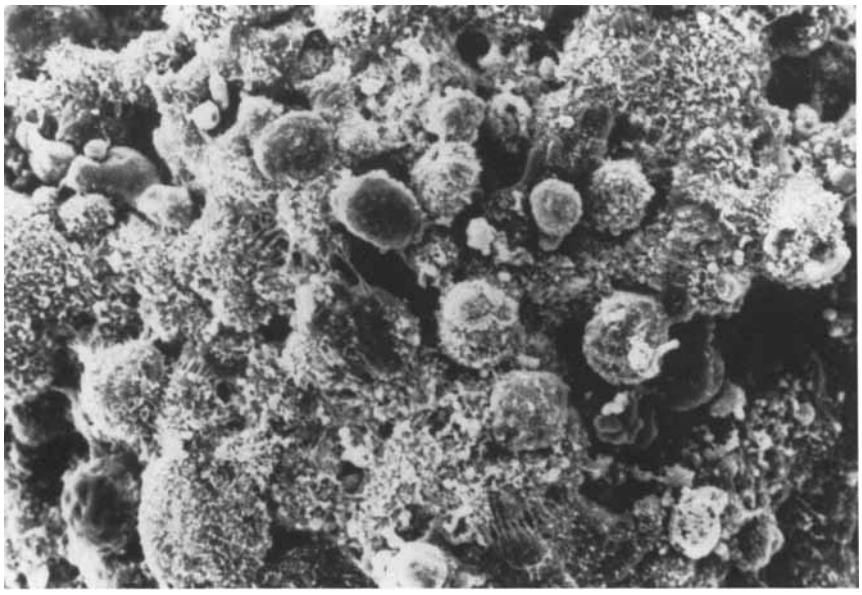

Fig. 3. Scanning electron micrograph of multicellular aggregate of LN1 cells cultured in the HARV Note the diversity of the cell populations present within the aggregate and the extensive development of surface microvilli (×750)

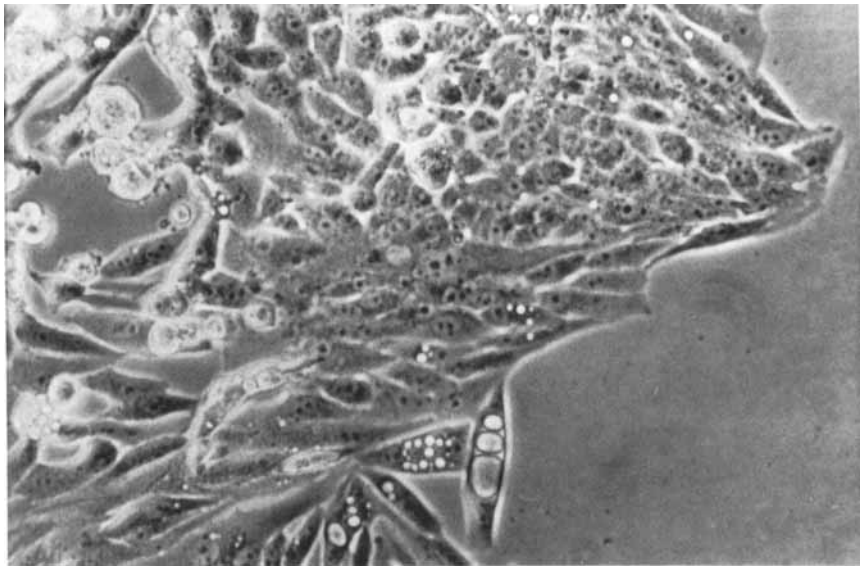

Fig. 4. Monolayer culture of LN1 cells recovered from the HARV (phase contrast ×250) Note the multiple cell types present, including elongated spindle cells and sarcomatous cells with lipid accumulation (lower right) and nest of epithelial-like cells (upper right)

rian tumors of the ovary. Moreover, these data suggest that LN1 may indeed represent a pluripotent stem cell capable of giving rise to the multiple malignant cell types that comprised the original tumor specimen. The origin of such a stem cell has been a much debated topic, particularly in the ovarian form of these unusual tumors, but it is postulated to arise from ovarian surface epithelium [Dehner et al., 1971]. The epithelial surface of the ovary is a modified mesothelial tissue that is embryologically derived from primitive coelomic mesothelium. Ovarian surface epithelium shares this embryologic origin with mullerian mesothelium. This common lineage is believed to account for the similarities between mixed mullerian tumors

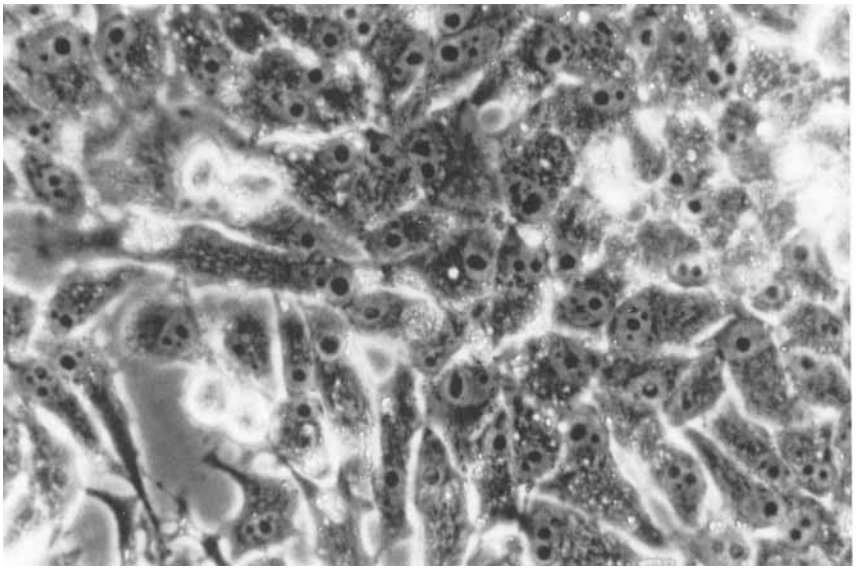

Fig. 5. Isolated focus of sarcomatous cells in monolayer culture of LN1 cells recovered from the HARV (phase contrast ×400) Note the polarized accumulation of osteoid material, indicative of chondrosarcoma differentiation

arising in the ovary and those of the uterine endometrium.

Early histological studies demonstrated that during the development of the müllerian duct from the urogenital ridge, epithelium and mesenchyme are so intimately associated that transitional cell forms exhibiting characteristics of both epithelial and mesenchymal cells are present [Gruenwald, 1943]. Lauchlan subsequently coined the term "secondary müllerian system" to refer to all müllerian-derived tissues located outside the cavities of the original müllerian ducts [Lauchlan, 1972]. Moreover, it was postulated that tissues derived from this coelomic epithelium and its accompanying mesenchyme have the potential to differentiate into müllerian-directed epithelium and stromal elements. It is noteworthy that ovarian surface epithelium comprises only a small fractional component of the ovary, but is the source of nearly all ovarian cancers [Blaustein, 1981].

Few studies have attempted to isolate a putative stem cell precursor from mixed müllerian tumors of the ovary. The first report on the generation of a long-term cell line from a homologous mixed müllerian ovarian tumor demonstrated that both epithelial and mesenchymal malignant components were maintained during serial transplantation in nude mice [Takeda et al., 1984]. Furthermore, the presence of transitional-type cells, from carcinomatous to sarcomatous, were frequently observed in the heterotransplants. This same cell line was subsequently cultured in vitro, resulting in the development of four clonal cell lines [Masuda et al., 1987]. Three of these clonal lines exhibited mesenchymal characteristics, whereas one line expressed a carcinomatous phenotype. Using low density cell passaging, a sarcomatous type cell line was found to arise from the carcinomatous line. In contrast, no sarcomatous to carcinomatous transitions were noted. The establishment of two additional cell lines, each derived from separate patients having homologous mixed müllerian tumors of the ovary, has recently been reported [van Haaften et al., 1988, 1990]. Although both lines showed epithelial differentiation, one cell line lost the ability to express simultaneously both vimentin and cytokeratin, which was present in the parent tumor. A vimentin-expressing subline was eventually derived from this vimentin-negative parent line, suggesting that the parent cell line was capable of multidirectional differentiation. It has been suggested that the coexpression of the cytokeratin and vimentin intermediate filaments indicates epithelial differentiation of müllerian-derived cells [Puts et al., 1987].

These studies demonstrate that the differentiation of LN1 induced by HARV culture is reversible. This is perhaps the most intriguing aspect of this work, for it suggests that a given cell has the capacity selectively to express a particular phenotype depending upon its culture condi-

tion. A theory applicable to this finding was proposed nearly 100 years ago by Schwalbe [1906] and reiterated in more recent years by Ober and Tovell [1959a]. According to the concept of "disontogenetic field," alterations in cell development can occur that are not necessarily manifested in the form of visibly different cells or groups of cells, but rather in the form of a potentiality to undergo neoplasia when appropriate conditions are achieved. This theory might be expanded to suggest that the growth potential of tumor cells cultured under appropriate conditions is limited only by the differentiation capacity of the tissue of origin. On this basis, the mullerian-derived LN1 cells grown in vitro under the "permissive" conditions provided by HARV culture might be expected to give rise to all the histological elements that arose from this tissue in vivo.

The question remains, what unique features of the HARV culture allow this type of cellular organization and differentiation? As described elsewhere, RWVs represent a nonstressful, low shear, low turbulence culture system that exerts minimal selective pressure on cell orientation [Schwarz et al., 1992]. In monolayer culture, cells are forced to grow in one plane under space-limiting conditions, resulting in an obvious artificial growth environment relative to development in vivo. In contrast, culture in the RWV promotes proliferation in three dimensions, allowing the cells to assume any orientation. Combined with conditions of minimal turbulence and shear, cells growing on separate carrier beads may associate to form bridges, thus enlarging the three-dimensional aggregate structure. Spacial orientation is critical to the differentiation capacity of the growing aggregate of cells. In this regard, the ability of spacial configuration to influence tumor glycoprotein expression has been described. Growth of LS-174T colon carcinoma cells as spheroids yielded enhanced expression of high molecular weight, mucin-like, carcinoma-associated glycoprotein (TAG-72), relative to cells grown in monolayer culture [Johnson et al., 1986]. Previous work in RWV culture has also demonstrated that three-dimensional aggregates of HT-29KM colon carcinoma cells exhibit increased synthesis of mucinous material, coincident with the development other structural indications of cellular differentiation [Goodwin et al., 1992].

FUTURE DIRECTIONS

The development of new treatment strategies and improved methods for the earlier detection of ovarian cancer requires a reproducible model for studying the growth and metastatic potential of these aggressive tumors. We present evidence in support of such a model. HARV culture of LN1 ovarian tumor cells resulted in the formation of three-dimensional cellular aggregates that were composed of multiple cell types, as existed in the original tumor specimen. Future studies will evaluate the usefulness of this model in testing tumor sensitivity to a particular chemotherapeutic agent or biological therapy. This type of in vitro "trial" will be particularly beneficial in determining the effectiveness of new forms of treatment. The RWV model of three-dimensional cell culture will also be used to examine alterations in gene expression, as a function of stage of tumor cell aggregate growth. These data will be applicable toward refining molecular-based approaches for the determination of genetic markers which may be useful in patient prognosis, as well as in designing specific targeted molecular therapies.

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