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# Cytometric Analysis of Neoplastic Transformation of Vertebrate Cell Populations

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## SUMMARY

Two parameters of neoplastic transformation in spontaneously transforming fibroblasts were examined. One parameter, the predilection for rounding of cells at metaphase, was studied using time-lapse cinephotomicrography and fixed, stained preparations. Ten different cell lines were assayed, including established lines of hamster, rat, and mouse cells, and six rodent cultures of known neoplastic potential as determined by animal injection and tumor production. There was a close correlation between the assumption by cells of a spherical shape at metaphase and their ability to form tumors on injection in syngeneic hosts. The projected area of adherent cells 24 hr after plating over a coverglass was used to assay the transition of these rodent cell populations in culture from nonneoplastic to neoplastic. As the cell population became neoplastic, there was a significant decrease in the mean projected area of the cells. Furthermore, as the cell cultures became capable of producing tumors, the projected area profile of the population shifted proportionally to smaller area classes.

## INTRODUCTION

Neoplastic transformation in cultured fibroblasts is accompanied by a variety of morphological changes (1, 8–10). One of the most obvious alterations is the retraction of cells from the surface of the substrate, a feature that has been observed in many cell cultures (of different animal origins) that have become neoplastic as the result of a variety of inducing agents (1).

This report quantifies 2 aspects of this phenomenon. First is the tendency of cells in neoplastic populations to assume a spherical shape at metaphase when grown on a glass or plastic substrate. Second is the decrease in projected area of fibroblasts growing on solid substrates as the cell populations develop a greater potential for growing as malignant neoplasms upon injection into suitable animals.

## MATERIALS AND METHODS

**Cell Populations Studied.** The cells used for projected area measurements were NCTC line 5067 originating from minced 11-day embryos of the ALB/N rat (6), NCTC clone 7914, and NCTC clone 7915, both from a cell line initiated from a mince of 12-day C3Hf/HeN mouse embryos. During their continuous propagation, all 3, cell line and 2 clones, were repeatedly assayed in syngeneic animals for evidence of neoplastic transformation (*i.e.*, growth as sarcomas), and all were judged to have undergone spontaneous transformation at some point. Assays were made by implantation of grossly visible compacted cell sheets at least 1 cu mm into the anterior chamber of the eye, an immunologically privileged site, or by i.m. injection of quantified cell suspension into animals X-irradiated (425 R) to reduce immune defenses. At suitable intervals, representative line or clone cultures were cryopreserved in liquid nitrogen.

For this study, 3 ampuls of NCTC 5067 were thawed and cultured in Medium NCTC 135 with 10% FBS[1] (Flow Laboratories, Rockville, Md.) for 49 days, or 6 transplant generations, before use. Cells of these lines were subcultured (1:2) when they formed a confluent sheet, usually at weekly intervals. The total *in vitro* growth periods of the cell populations from the 3 ampuls were 126,280, and 847 days (10, 17, and 50 transplant generations), respectively. Line 5067 cells implanted intraocularly had produced no tumors in 30 rats after 56 to 91 days *in vitro* or in 25 rats assayed between 154 and 252 days, but by 797 days all of 10 implanted rats developed sarcomas (6). By this criterion, the 1st 2 cultures were considered nonneoplastic, while the last was considered definitely neoplastic.

Two ampuls of Clone 7914 were used. Cells from 1 ampul were grown in Dulbecco-Vogt's modification of minimum essential medium for 14 days before use. Cells from a 2nd ampul were cultured in Medium NCTC 135 plus 10% FBS for 63 days before use. A 3rd subline of the clone, never subjected to cryopreservation, was also grown in NCTC 135 plus 10% FBS. The total *in vitro* growth periods for these 3 lines were 344, 364, and 1006 days (31, 33, and 102 transplant generations), respectively. Of the 3, only the cultures grown continuously for up to 1006 days produced tumors in mice. This line produced tumors in 23 out of 23 animals given i.m. injections of $10^5$, $10^6$, or $10^7$ cells that had been grown for 757 days in culture. Earlier inoculations were made by intraocular or i.m. implantation of 1 to 2 × $10^6$ cells/animal.

Three ampuls of Clone 7915 were thawed and the cells were cultured in Dulbecco-Vogt's modification of minimum essential medium plus 10% FBS for 20 to 24 days before

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[1] The abbreviation used is: FBS, fetal bovine serum.

use. The total *in vitro* growth periods were 277, 353, and 543 days (22, 45, and 92 transplant generations), respectively. This clone produced no tumors in 4 mice given i.m. injections of $10^6$ cells at 273 days cultivation, but produced tumors in 4 of 5 mice implanted intraocularly after 376 days cultivation, and in 5 of 5 mice implanted intraocularly after 474 days of *in vitro* cultivation. By this criterion, the 1st culture was considered nonneoplastic, the 2nd culture was considered borderline neoplastic, and the 3rd culture, fully neoplastic.

The cell lines used for the cinephctographic portions of this study are briefly described in Table 1.

**Methods of Measuring the Projected Area of Cells.** Cells from confluent cultures were dispersed with a Versene-trypsin solution, ATV (7), and inoculated into Leighton tubes containing acid-cleaned 9- × 50-mm coverslips (No. 1; 0.15 mm thick). The inoculum consisted of 2 ml of medium containing $5 \times 10^3$ cells/ml. The tubes were incubated for 24 hr at 37°. Cells were fixed in 3% glutaraldehyde followed by dehydration in graded alcohols and embedded in index of refraction medium ($n_D$ 1.400, R. P. Cargille Laboratory, Cedar Grove, N. J.). The cells were photographed in field interference contrast (Fig. 1*A*) with a Leitz Mach-Zehnder double-beam interference microscope. This microscopy technique was used for the visualization of the cells, as the image does not suffer from halo or "shadowing" such as occurs with phase contrast and differential interference microscopy techniques.

The photographic image (recorded on SO-410 35-mm film; Eastman Kodak, Rochester, N. Y.) was enlarged to a final magnification of × 1000, and an outline drawing was made of the cell periphery. The projected area of individual cells was obtained with a previously described (4, 5) video integrator system. In all but 1 case, the projected area of more than 200 cells/sample was measured. Care was taken to ensure that only isolated, well-resolved cells were measured.

**Measurement of Mitotic Rounding.** Two methods of observation were used for measurement of mitotic rounding. First, mitoses of cells grown in this laboratory and photographed with time-lapse cinephotomicrography (ranging in time from 1949 to 1975) were studied in cases in which the cells were recently isolated from normal tissues or had been established as continuous lines, and their oncogenic potential was determined by animal injection. Metaphase was established by film analysis on a Vanguard 16-mm film analyzer or, in the case of older films, on a 35-mm Movieola film editor. A total of 22 films were analyzed. In addition, preparations of Leighton tube cultures were fixed after 3 days of incubation and stained with Giemsa, Feulgen-light green, or with gallocyanin-chrome alum-naphthol yellow S. Carnoy's fixative was used as a control for Feulgen-stained preparations. In addition, cell cultures were fixed in 3% glutaraldehyde containing 1% deionized formalin, buffered with phosphate to pH 7.3 in normal 0.85% NaCl solution. This non-coagulating fixative gave distinctly superior results, and cell shrinkage was determined to be less than 5%, when comparing cultures before and after fixation.

Rounding of metaphase figures was rated on an arbitrary 5-point scale that is described in Table 1. This rating system was applied to both fixed and cine images of dividing cells.

## RESULTS

Projected cell area was determined in 3 rodent cell line populations as they progressed toward becoming neoplastic. With increasing time in culture there was a corresponding decrease in the projected area of these cell populations. Chart 1 shows the projected cell area profiles of NCTC 7915 at 3 different points in its cultural history. After 277 days in culture (Chart 1, *top*) no animals given injections of NCTC 7915 developed tumors. The mean projected cell area was 7107 sq $\mu$m, with a normal distribution of areas. After 353 days in culture, *i.e.*, in the period between the last non-tumor-producing injection and the 1st injection that produced tumors in 4 out of 5 animals (at 376 days in culture; Chart 1, *middle*), the projected cell area profile for this cell line shifted toward smaller values,

Table 1

*Metaphase morphology from cinephotomicrographic studies*

| NCTC Cell line | Cell source | Neoplastic (+) or non-neoplastic (0) | No. of days *in vitro* | No. of metaphases observed | Metaphase morphology (% cells) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | 1[a] | 2 | 3 | 4 | 5 |
| 3209 | Mouse thymus | 0 | 195 | 60 | 65 | 13 | 5 | 13 | 0 |
| 4118 | Syrian hamster embryo | 0 | 9 | 20 | 35 | 60 | 0 | 5 | 0 |
| 5203 | Syrian hamster embryo | 0 | 15 | 31 | 55 | 40 | 0 | 6 | 0 |
| 5454 | Rat embryo | 0 | 28 | 58 | 29 | 40 | 7 | 12 | 12 |
| 8322 | Mouse embryo | 0 | 293–362[b] | 223 | 39 | 19 | 3 | 19 | 20 |
| 3631 | Mouse embryo | + | 162 | 123 | 0 | 2 | 0 | 7 | 92 |
| 4094 | Mouse parotid | + | 202 | 15 | 0 | 7 | 0 | 7 | 87 |
| 3403 | Mouse embryo | + | 449 | 75 | 0 | 0 | 0 | 0 | 100 |
| 2445 | Mouse connective tissue | + | 5321 | 15 | 0 | 0 | 0 | 0 | 100 |
| 2071 | Mouse connective tissue | + | 670 | 73 | 0 | 0 | 0 | 4 | 96 |
| 7943 | Mouse embryo | + | 540–737[b] | 218 | 4 | 2 | 1 | 15 | 78 |

[a] 1, spread metaphase; 2, oval metaphase with 2 or more cytoplasmic attachments to substrate; 3, oval metaphase with less than 2 cytoplasmic attachments to substrate; 4, round metaphase with 2 or more cytoplasmic attachments to substrate; 5, round metaphase with 1 or more apparent attachments to substrate.

[b] Combined data from 5 films.

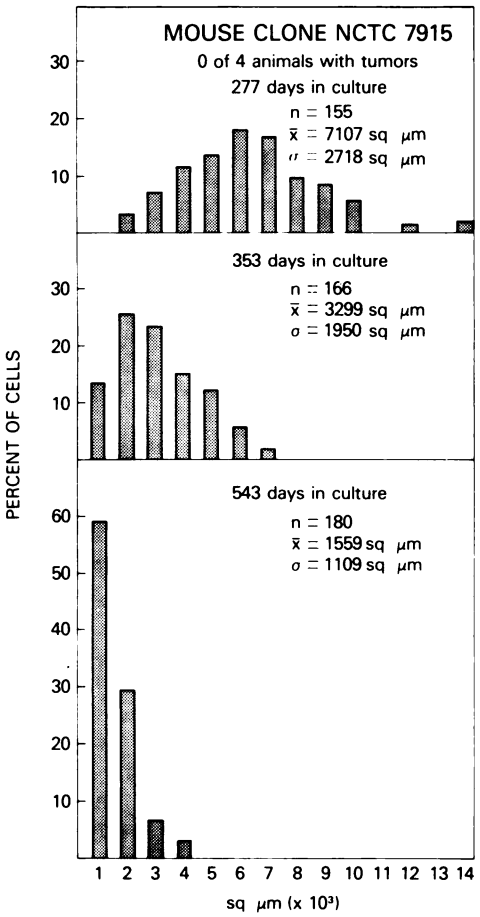


Chart 1. Histogram of projected area profile of mouse clone 7915 at 277, 353, and 543 days of culture. *n*, number of cells measured; $\bar{x}$, mean projected area; and $\sigma$, S.D. Cultures at 277 days produced no tumors on injection. However, after 543 days in culture, all animals given cell injections produced tumors.

with the shift being statistically significant ($P \leq 0.001$). Chart 1, *bottom*, shows the distribution of projected areas after the cells had been cultured for 543 days, at which point all animals that had been given injections developed tumors with short latent periods. The shift in the population to progressively smaller projected cell areas has become more pronounced, and the overall distribution has assumed the character of a $\gamma$ function.

Chart 2 shows the results of analysis of Clone NCTC 7914. Cultures at 344 days *in vitro* failed to produce tumors when injected into animals as did cells injected after 364 days. There is no significant difference between the projected cell area profiles of the 2 populations. After 1006 days, the projected area profile of the cell population had shifted and there was rapid tumor production in 23 out of 23 animals.

Chart 3 shows the results of analysis of a population of rat fibroblasts designated NCTC 5067. At 126 days in culture there was wide dispersion in the projected area profile of the cell population. These cultures did not produce tumors upon injection into suitable hosts. However, after 847 days in culture, there was a shift toward a decreased projected cell area significant at the $p \leq 0.001$ level and all animals that received injections promptly produced tumors.

Metaphase rounding was a predominant event in cell populations that were proven oncogenic by animal inoculation. Table 1 shows the incidence of spread and rounded metaphases in the 10 cell lines studied. Typical examples of each rating classification are shown in Fig., *B* to *F*. Several independent observers were able to use this classification system with no substantial disagreement.

## DISCUSSION

The decrease in projected cell area and rounding of rodent fibroblasts at metaphase may be related phenomena and may reflect a fundamental difference between cell populations that produce tumors on injection into suitable animals and those that do not.

According to Cherny *et al.* (2), the decreases in projected

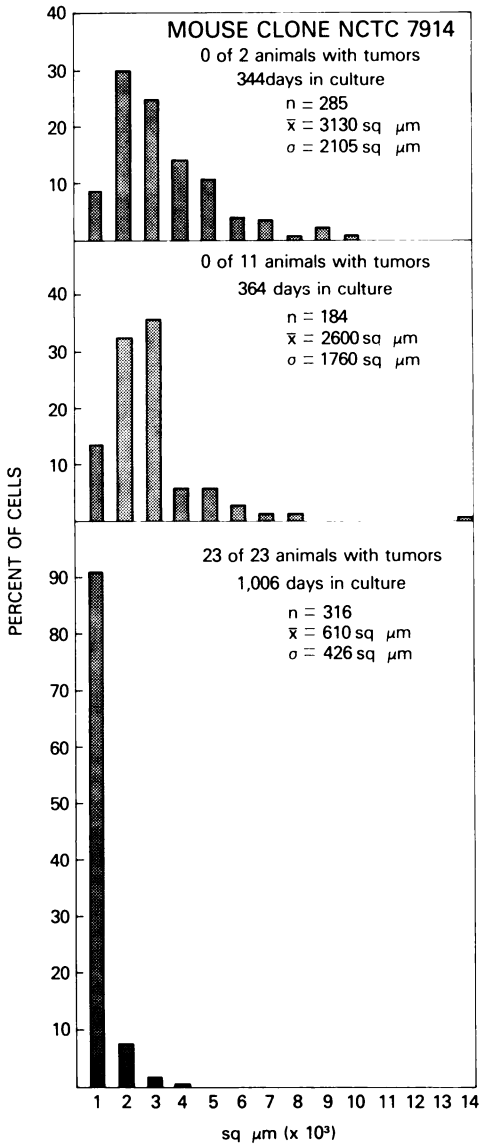


Chart 2. Histogram of projected area profile of mouse Clone 7914 at 285, 364, and 1006 days of culture. No tumors were produced after 344 or 364 days in culture. However, after 1006 days in culture, all animals given injections of cells developed tumors with short latent periods.

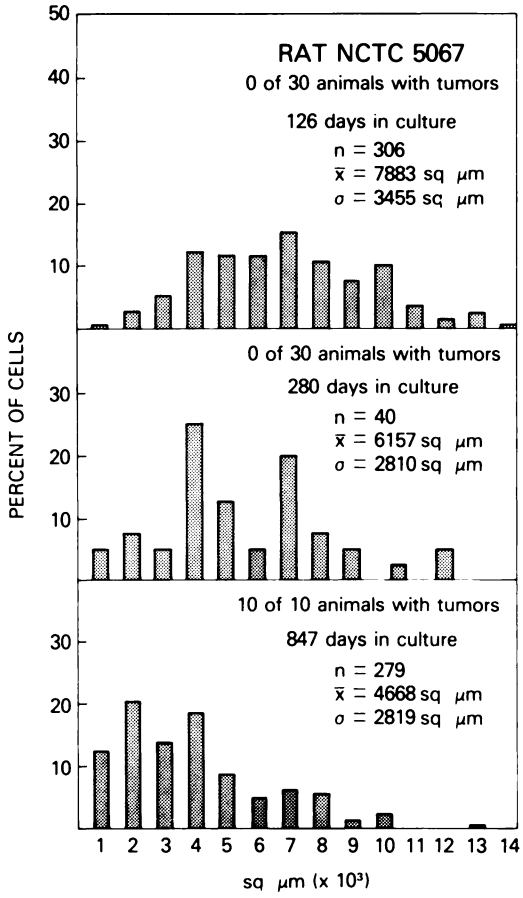


Chart 3. Histogram of projected area profile of rat fibroblasts (NCTC 5067) at 126, 280, and 847 days of culture. Note the broad spread of projected area profiles of the cells.

cell area that we report here progressing with development of oncogenic potential are due to a decrease in "lamellar cytoplasm," that is, the peripheral cytoplasm of the spread fibroblast which constitutes much of the area of nonneoplastic, fully spread cells. They have found that there is a decreased projected cell area of transformed mouse fibroblasts *in vitro*, and the lack of cell spreading seems to be independent of cultural conditions or cell density.

The work reported here confirms and extends the report of Cherny *et al.* (2). However, there may be other mechanisms by which such a phenomenon may arise. For example, the lamellar cytoplasm is very thin, as revealed by electron microscopy (2), and seems to consist of small amounts of cytoplasmic material between 2 layers of cell membrane. The amount of actual cytoplasm contained in this material is fairly small as revealed by interference microscopy of spread cells. The principal difference between oncogenic and nononcogenic cells lies in the amounts of cell membrane elaborated to form the covering of the lamelloplasmic extensions. If there is no difference in the amounts of cell membrane produced by oncogenic and nononcogenic cells, then it follows that oncogenic cells may have a large amount of membrane stored at their upper surface in the form of microvilli or other cell projections. However, in a scanning electron microscopic study of microvilli on the surface of neoplastic and nonneoplastic cells, including Clones NCTC 7914 and 7915, we were unable to detect a relationship between microvillus formation and oncogenicity of cell cultures (C. H. Fox, J. A. Dvorak, K. K. Sanford, and B. K. Wetzel, unpublished observation). Neither was there a correlation between growth rate and formation of microvilli other than during the active process of mitosis.

Until the scanning electron microscopic observations can be quantified, we hypothesize that a major factor in the formation of lamellar cytoplasm is the formation of new cell membrane and that this production in neoplastic cells is abridged in comparison to nonneoplastic cells. This elaboration of new membrane may progress to some fixed point in the cell cycle, after which the projected area of the cells does not increase. This phenomenon may be genetically controlled.

Possibly, a decrease in the amount of lamellar cytoplasm is a reflection of the transition rate through different portions of the cell cycle. Production of membrane material and thus of lamellar cytoplasm may cease after a certain point in the S or $G_2$ phase so that, if a cell suffered impeded or slow synthesis of membrane, the critical point of cell division would be reached before the cell had reached its full dimensions. This hypothesis is amenable to experimental verification.

The metaphase rounding phenomenon is due to the fact that as the cell enters mitosis there is a release of the cell periphery so that the cell remains attached to the substratum at only a few points. This retraction from attachment is most marked at metaphase in cell populations that have become neoplastic. It is not an absolute indicator of neoplastic state, however, because in most cultures an occasional cell may divide with either rounded metaphase in normal cultures, or spread metaphase in neoplastic cultures (*cf.* Table 1, NCTC 4094 and NCTC 7943). The retraction of a cell may be due to several factors, such as the original number of attachment points to the substrate, the ease with which cellular attachments may be formed or released, the surface area of the cell, including the amounts of cell membrane involved, and probably endoskeletal activities of the cell such as microtubular arrays and other filamentous structures.

We would like to emphasize that, in the phenomena of rounding at metaphase and reduced lamellar cytoplasm, basic morphological differences between normal and neoplastic cells initially derived from the same cell are observed. These differences are the reflection of biochemical events that can be studied with currently available technology.

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Fig. 1. *A*, interference photomicrograph of a cell from Clone NCTC 7915 showing the type of image used for projected area profile analysis. The *white area* in the center of the cell denotes a thickened region in which the optical retardation is in excess of one-half wavelength (546 nm). In *B* to *F*, are examples of cells used to assay metaphase rounding, as shown in Table 1. *B*, typical spread metaphase of nonneoplastic cells. The cell is flattened and attached at a number of points; *C*, more retracted type of metaphase in which the cell is attached at each end, but the cytoplasm is essentially oval; *D*, oval mitotic cell with only 1 apparent attachment; *E*, rounded cell with 2 or more attachments; *F*, completely rounded cell with 1 attachment or less. *Bar*, 20 $\mu$m.