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In Vitro and *in Vivo* Growth Characteristics of Two Different Cell Populations in an Established Line of Human Neuroblastoma[1]

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ABSTRACT

Two distinct cell morphologies were appreciated and separated in a long-established culture line (CHP-100) of human neuroblastoma. Both cell types carried chromosomal markers characteristic of neuroblastoma cells and the parent line; in addition, separate karyotypic changes in each cell type established them as separate and enriched populations. A small, refractile cell, designated CHP-100-S, was present and formed numerous cytoplasmic processes. A distinctly larger cell, CHP-100-L, was less refractile and lacked processes. The two cell types exhibited marked differences in adhesive properties *in vitro*. CHP-100-L adhered tightly to the culture flask and required enzymatic treatment for removal; CHP-100-S adhered loosely and could be harvested into the medium by simply tapping the flask. These two harvesting procedures were used to obtain highly enriched populations of each cell type, both of which proved to be tumorigenic in the nude mouse. *In vitro*, no significant difference in growth rates was observed between CHP-100-S (doubling time, 26 hr) and CHP-100-L (21 hr). However, in the nude mice, following inoculation of equal cell numbers, CHP-100-L cells grew much larger tumors than did CHP-100-S cells (3- to 100-fold increases over 25 days). Local invasion was also noted more frequently with the CHP-100-L explants. Reculturing of the mouse explants showed that the distinct cell morphologies were maintained even after multiple passages. The presence of heterogeneous cell populations in single tumors is of much potential importance for the clinical and biological behavior of neoplasms. The present data establish cultured human neuroblastomas as one model for studies of cell heterogeneity and suggest potentially important ramifications for the different cell types observed in the growth patterns of this neoplasm.

INTRODUCTION

The heterogeneity among cell populations in solid tumors has been increasingly recognized and characterized (3, 6, 7). The existence of this heterogeneity bears much potential importance to the clinical behavior of tumors (6, 7), to the distribution of biochemical markers in neoplastic tissue (3), and to tumor response to different treatment modalities (1, 6). Neuroblastoma is an important neoplasm to study in terms of its constituent cell populations. This tumor exists in a spectrum of neural differentiation, ranging from undifferentiated neuroblasts to mature ganglion cells. In the present study, we report the characterization of 2 cell populations which we observed in a long-established culture line of human neuroblastoma. The *in vitro* behavior of the 2 cell types is compared to their *in vivo* growth patterns in the athymic (nude) mouse. Different morphological and adhesive properties observed, *in vitro*, appear to have important ramifications for the tumorigenicity of the cells.

METHODS

Materials. Cell culture consumables including medium (Roswell Park Memorial Institute Tissue Culture Medium 1640), fetal calf serum, antibiotics (penicillin and streptomycin), and bicarbonate, and glutamine were obtained from Grand Island Biological Co. (Grand Island, N. Y.). L-[^{14}C]Dopa,[4] 8 mCi/mM, was purchased from Amersham/Searle Corp. (Arlington Heights, Ill.). Athymic nude mice (*nu/nu*) of BALB/c background were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, Mass.).

Cell Culture. The CHP-100 line of human neuroblastoma cells (9) was a gift from Dr. Herbert Kaizer of the Johns Hopkins Oncology Center; the line was obtained from Dr. Audrey Evans of the CHP, Philadelphia, Pa. All cells were grown in 75-sq cm flasks and maintained in 20 ml of Roswell Park Memorial Institute Tissue Culture Medium 1640 with 100 units of penicillin, 100 µg of streptomycin, 2 mM glutamine supplementation, and 16% heat-inactivated fetal calf serum at 37° in 5% CO_2 atmosphere. The medium was changed every 2 to 3 days. Cell counts for growth curves were performed on a hemocytometer using trypan blue exclusion to assess viability. Cell harvesting was performed according to the morphology of the cell types as discussed in "Results." The cells were gently triturated in a pipet to produce single-cell suspensions in phosphate-buffered saline for cell counting. Phase microscopy photography was performed with a Zeiss inverted-phase microscope and a Polaroid camera attachment.

Karyotype Analysis. The karyotypes for the parent cells of CHP-100 and each of the 2 subpopulations currently investigated were analyzed by a modified trypsin-Giemsa protocol described previously (8).

Nude Mouse Inoculations. Each mouse for study received s.c., in each flank, a single injection of 1×10^6 viable cells prepared as for cell counting. Aliquots of the 2 cell populations studied were always injected into the same animal in opposite flanks. Tumor volume was calculated using caliper measurements. Five weeks after receiving injection, the mice were sacrificed and examined for final tumor size and invasion of adjacent structures. The excised tumors were mechanically dispersed and reestablished in culture under the conditions detailed above.

Enzyme Assays. L-Dopa decarboxylase activity was measured as described previously (2-4) using L-[^{14}C]dopa as substrate. Choline acetyltransferase activity was kindly measured by Dr. Barbara Talamo

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[4] The abbreviations used are: L-dopa, 3,4-dihydroxy-L-phenylalanine; CHP, Children's Hospital of Philadelphia.

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(Department of Neurology, The Johns Hopkins University School of Medicine) and dopamine β-hydroxylase by Dr. Reinhard Grzanna (Department of Cell Biology and Anatomy, the Johns Hopkins University School of Medicine).

RESULTS

Description of Cell Types. Two distinct cell forms were serendipitously observed in the neuroblastoma cell line, CHP-100, maintained under the growth conditions outlined in "Methods." The first cell type, labeled CHP-100-S, is more refractile, is distinctly smaller in cell body size, and has cytoplasmic extensions greater than 40 μm in length (Fig. 1*A*). A second cell type, labeled CHP-100-L, was larger and less refractile and had no cytoplasmic extensions (Fig. 1*A*).

CHP-100-S cells were noted to detach easily from the surface by a sharp tap of the culture flask, while CHP-100-L cells attached firmly to the flask surface and required treatment with Viokase for removal. CHP-100-S cells also tended to form dense clusters, while CHP-100-L cells formed a flat monolayer. The cell populations could be separated from one another by exploiting these different adhesive properties (see Fig. 1). The 2 enriched cell populations remained stable with respect to their attachment and morphological characteristics upon serial passaging in culture (Fig. 1, *B* and *C*).

Karyotype Analyses. Karyotypes of the 2 sublines (Fig. 2), CHP-100-S and CHP-100-L, have been compared with each other and with that obtained from the original CHP-100 cell line (9). All 3 lines had a near-diploid mode, with the chromosome number per cell for each line being 46 ± 3 (S.E.). All 3 were missing an X chromosome and contained the same marker chromosome 1, with intact long arms and a translocation at 1p32 (origin of the translocated segment unknown). Deletions and/or rearrangements of chromosome 1p involving the loss of structural material distal to 1p31 have been found in greater than 50% of all neuroblastoma tumors and cell lines analyzed; this has led to the proposal that the distal portion of 1p contains one or more genes responsible for the development of this particular cancer (8).

Line CHP-100-L shares with CHP-100 two additional rearrangements (16p+,q+ and a marker chromosome the components of which have not been identified); CHP-100-L also contains a 3p− and a 10q− chromosome (Fig. 2). Line CHP-100-S contains an isochromosome for the long arm of 17 [i(17q)] and varying numbers of small, paired chromatin bodies per cell, termed double minutes (Fig. 2). The fact that the 2 lines share chromosome markers in common with CHP-100 suggests that all are derived from a single progenitor. The fact that CHP-100-S and CHP-100-L cells can be distinguished from one another by virtue of the different chromosome abnormalities confirms the extensive enrichment of these cell populations and is consistent with the hypothesis that genetic heterogeneity can develop over time in continuously dividing cell lines.

***In Vitro* and *in Vivo* Growth Characteristics of the Cell Types.** The growth rates of the 2 enriched cell populations are shown in Chart 1. No significant difference in doubling time (26 hr for CHP-100-S *versus* 21 hr for CHP-100-L) was observed between the 2 cell types *in vitro*.

Both cell types formed tumors when implanted s.c. into nude mice. The growth curves in Chart 2 and the tumor masses in Fig. 3 show that the CHP-100-L cells grew much more rapidly in the nude mouse than did a similar inoculum size of CHP-100-S cells. Upon retransfer of the cell populations from the nude mice back to cell culture, the cell morphology and adhesion features remained unchanged even after repeated passages in the mouse (Fig. 4). The *in vivo* growth rate difference, however, was not maintained *in vitro* (Chart 1); inoculation back into the nude mouse again demonstrated the *in vivo* difference in growth rates. The tumors formed with CHP-100-L cells also tended to show early invasion of surrounding structures even when compared to similar sized tumors formed with CHP-100-S cells.

Biochemical Properties of the Cell Types. The biochemical

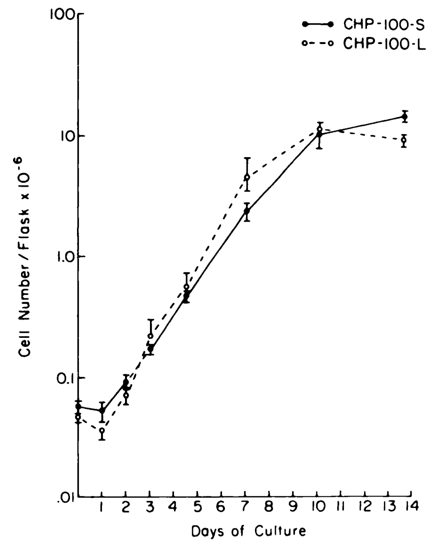

Chart 1. Growth curves for cultures of CHP-100-S and CHP-100-L cells. Cell counts are an average of 3 determinations from 3 separate experiments. Both the CHP-100-S and CHP-100-L cells had been passaged once in a nude mouse as described in the text; results were identical to those with cultured cells not passaged in the mouse. *Bars*, S.E.

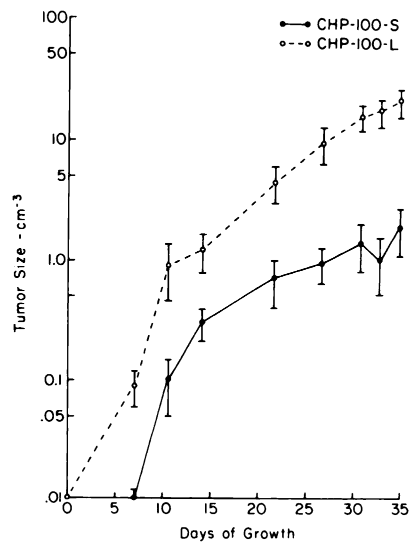

Chart 2. Growth curves for nude mouse explants of CHP-100-S and CHP-100-L cells. Inoculations and tumor mass measurements were performed as outlined in "Methods." The experiment shown involved 7 of 8 animals which survived the 5-week study period. The results are identical to those of 4 other experiments which were performed. Results are averaged for 3 CHP-100-L tumors and 4 CHP-100-S tumors; *bars*, S.E.

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Table 1
Neurotransmitter-synthesizing capacity of CHP-100-S and CHP-100-L cells

	L-Dopa decarboxylase	Dopamine β-hydroxylase	Choline acetyl transferase (× 10^5 dpm/ 10^{-6} cells)[a]
CHP-100-S	0	0	1.8
CHP-100-L	0	0	2.3

[a] Counts in labeled acetylcholine formed from labeled acetylcoenzyme A substrate.

characteristics of the 2 cell types are outlined in Table 1. High and similar specific activities of the cholinergic enzyme, choline acetyltransferase, were observed in both cell types; activities of the biogenic amine-synthesizing enzymes, L-dopa decarboxylase and dopamine β-hydroxylase, were nonmeasurable in both. This high cholinergic activity and absent biogenic amine-synthesizing capacity were exactly as described in the initial report of the CHP-100 line (9).

DISCUSSION

We have characterized 2 distinct cell types in a well-established human neuroblastoma cell line that differed in their *in vitro* phenotypic properties. Both cell types carry karyotype markers of the parent cell line and distinctive markers of their own, indicating the excellent enrichment obtained by manipulating their different adhesive properties. Review of the literature reveals that the morphological features of the cell populations we have identified have been observed in other culture lines of human neuroblastoma. Biedler *et al.* (5) recognized, but did not functionally characterize, 2 populations of cloned cells related by karyotype pattern to a common precursor cell in neuroblastoma line Sk-N-SH (5). Their "epithelial-like" clones closely resemble the morphology and anchorage dependency of our CHP-100-L cells, and their "neuroblast" clones were similar to our CHP-100-S cells. Thus, the behavior of cell types we report may have general relevance to populations of neuroblastoma cells.

Most importantly, we have documented that despite similar growth rates *in vitro*, our 2 cell types manifest distinctly different growth characteristics *in vivo* (implanted into nude mice); the flat cell type, CHP-100-L, grows much faster. It also appeared that CHP-100-L is more invasive than CHP-100-S and demonstrates early penetration into the muscle layers and the peritoneal cavity of the mouse as compared to lack of such behavior by CHP-100-S cells at similar tumor sizes. Thus, differences in biological characteristics were observed between the 2 cell types *in vivo* that could not be appreciated in the *in vitro* cultures. The transfer of cell types between a culture system and an *in vivo* carrier, such as the nude mouse, constitutes one valuable model system for full characterization of the biological behavior of heterogeneous cell populations of human tumors.

The specific properties of neuroblastoma cells that account for the spectrum of aggressive behavior that they exhibit in humans have not been characterized. How these cell properties may relate to their state of differentiation is also not clear. We feel that the study of cell types in cultured human neuroblastomas, which differ in their morphological and biological behavior, may provide critical information in this regard. Such systems could help identify differentiation characteristics which correlate with tumorigenicity and metastatic behavior. Also, the differential drug sensitivities of identified cell populations might be obtained. The heterogeneity of human tumors is an important biological aspect of neoplastic growth; investigations of the subpopulations of cells in human neuroblastoma provide another potentially important model for extending our understanding of this phenomenon.

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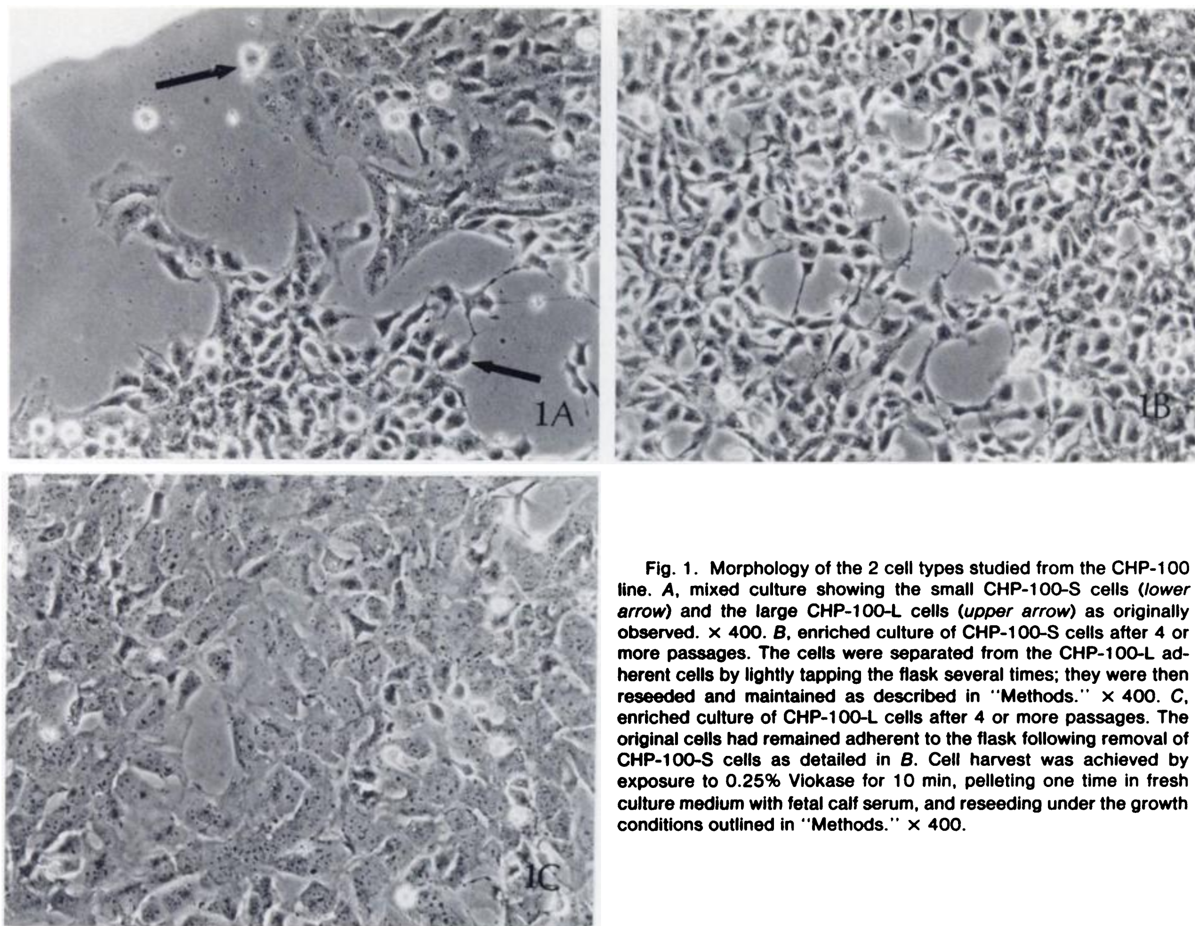

Fig. 1. Morphology of the 2 cell types studied from the CHP-100 line. *A*, mixed culture showing the small CHP-100-S cells (*lower arrow*) and the large CHP-100-L cells (*upper arrow*) as originally observed. × 400. *B*, enriched culture of CHP-100-S cells after 4 or more passages. The cells were separated from the CHP-100-L adherent cells by lightly tapping the flask several times; they were then reseeded and maintained as described in "Methods." × 400. *C*, enriched culture of CHP-100-L cells after 4 or more passages. The original cells had remained adherent to the flask following removal of CHP-100-S cells as detailed in *B*. Cell harvest was achieved by exposure to 0.25% Viokase for 10 min, pelleting one time in fresh culture medium with fetal calf serum, and reseeding under the growth conditions outlined in "Methods." × 400.

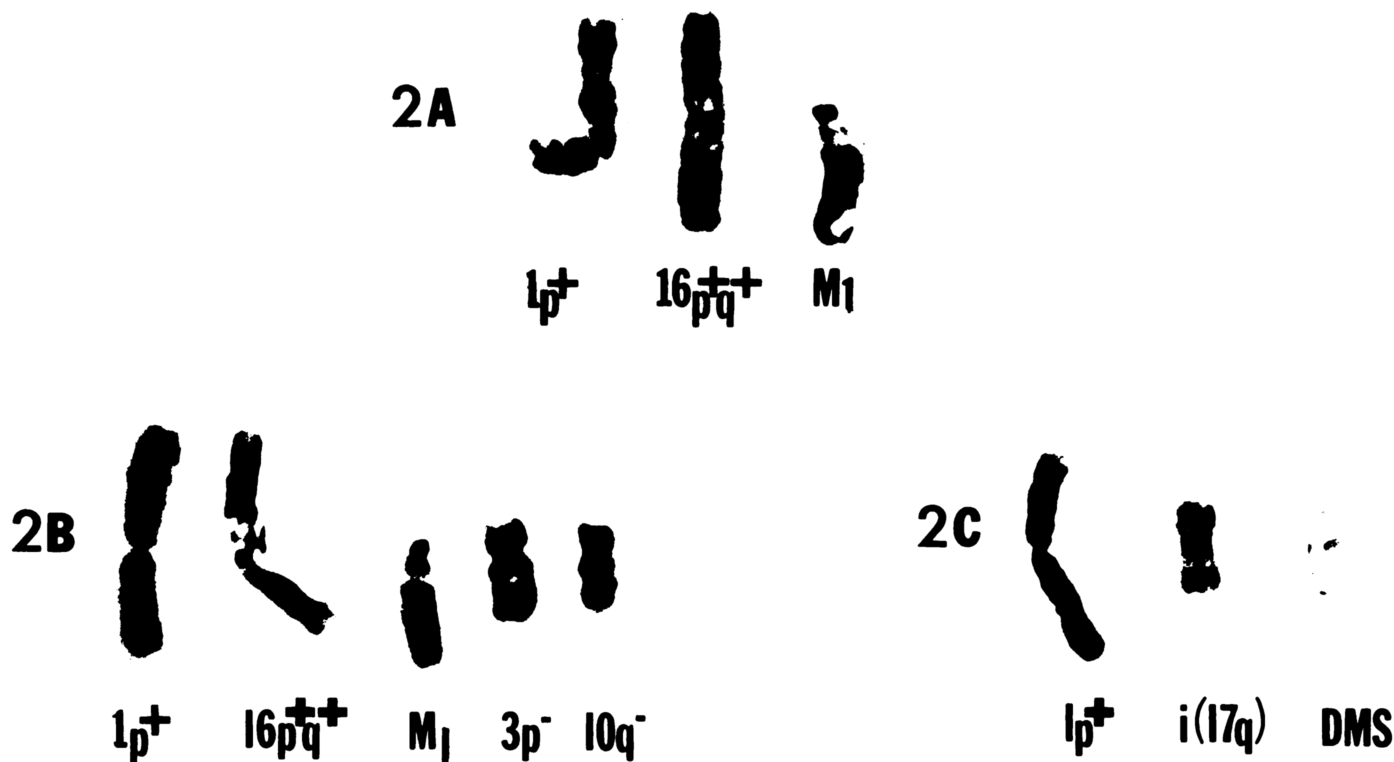

Fig. 2. Karyotypic markers for the parent line of CHP-100 and the sublines, CHP-100-L and CHP-100-S. *A*, CHP-100 parent line. Marker rearrangements identified: 1p+; 16p+,q+; and M1 (a marker whose origin cannot be further defined). *B*, CHP-100-L. Marker rearrangements include: 1p+; 16p+,q+; 17, etc., and M1 with 2 additional consistent rearrangements, 3p− and 10q−. *C*, CHP-100-S. Note presence of the same 1p+ as in CHP-100 and CHP-100-L; also a new rearrangement, iso(17q); and varying numbers of double minute chromosomes (*DMS*) per cell.

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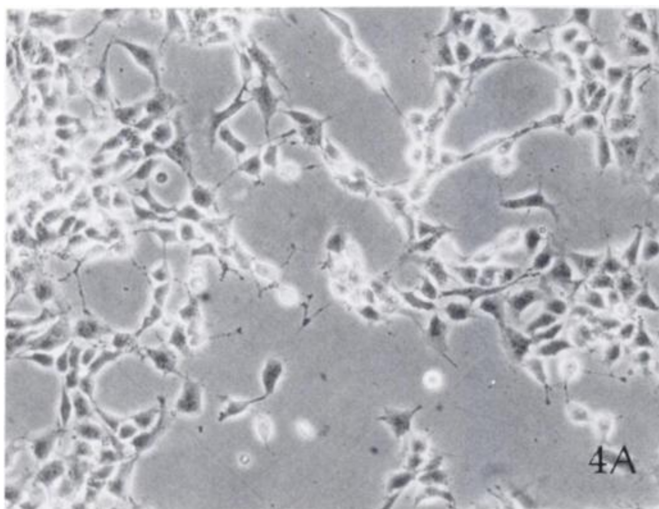

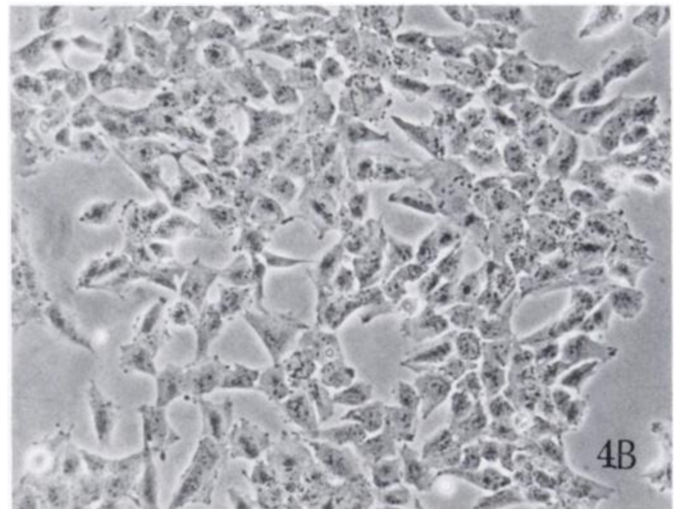

Fig. 3. Athymic nude mouse with equal numbers (1×10^8 viable) of CHP-100-S cells (*right side*) and CHP-100-L cells (*left side*) injected 35 days prior to photograph. *Inset*, 2 separate animals.

Fig. 4. Culture morphology of the 2 cell populations harvested from nude mouse explants. Culture conditions were identical to those for Fig. 1. × 400. *A*, cells from a nude mouse explant of CHP-100-S cells after 4 passages in culture; *B*, cells from nude mouse explant of CHP-100-L cells after 4 passages in culture. The morphology of each cell population appears identical to those seen in Fig. 1.

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