Eur J Cancer Clin Oncol, Vol. 20, No. 12, pp. 1517–1526, 1984.
Printed in Great Britain.

0277-5379/84$3.00+0.00

Increased Metastatic Ability and Bone Formation of a Mammary Adenocarcinoma *in Vivo* after *in Vitro* Passaging*

DAVE B. HOON,†‡ HSI-CHANG WANG§ and IAN A. RAMSHAW||

†*Department of Surgical Oncology, UCLA School of Medicine, CHS54140, Los Angeles, CA* 90024, *U.S.A.*, §*Department of Anatomy, College of Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 0W0 and* ||*Department of Experimental Pathology, John Curtin School of Medical Research, Australian National University, Canberra City, Australia A.C.T.* 2601

Abstract—*On* in vitro *passaging of the rat mammary adenocarcinoma R3230AC cell line, phenotypic changes occurred that were expressed* in vivo. *The histology of this mammary adenocarcinoma changed to a fibrosarcoma and then to an osteosarcoma. The overall population of early passaged cells consisted of a mixture of predominantly epithelial-like cells and few fibroblast-like cells; however, later passaged cells consisted more of the latter type. Along with the changes in histology, the tumor line also became highly metastatic in animals after* in vitro *passaging. The etiology of these phenotypic changes was not determined. Cytogenetic studies revealed chromosome changes in cells of later passages. Cells of* 90 *or greater passages that produced bone tumors were found to have fewer chromosomes and a metacentric marker isochromosome. In this report a correlation is made between the aberrant change in histology, increased metastatic ability and the presence of a marker chromosome of the R3230AC tumor.*

INTRODUCTION

THE PHENOTYPIC expressions of a tumor are continuously changing during tumor progression [1]. The mechanisms by which these changes occur is not understood. However, it is thought that genetic or epigenetic factors or both are responsible [2]. Nowell [3] suggests that tumor progression is a consequence of the genetic instability of tumor cells, whereby new clones of different phenotypes are produced. The process of selection of these clones is one mechanism of tumor progression. Because the cellular environment within a tumor is abnormal, there is an existing pressure for selection of unusual tumor cell phenotypes [2]. Similarly, the environment of tumor cells in *in vitro* may also exert a similar effect for selection of aberrant tumor cell phenotypes [4, 5]. There are reports of well-differentiated tumor cell lines becoming poorly differentiated tumor lines after either *in vitro* or *in vivo* passaging [4–9]. Along with phenotypic changes during tumor progression, cytogenetic changes can also occur [10–12]. However, the direct association of cytogenetic changes to phenotypic changes can be found in only a few tumors [13]. In the course of our investigation of the mammary adenocarcinoma R3230AC, we found that on *in vitro* passaging the tumor's phenotypic expression *in vivo* changed. The R3230AC line at early passages was classified as a differentiated mammary adenocarcinoma, but through continuous *in vitro* passaging (>50) the histology of the tumor changed to a fibrosarcoma and later to an osteosarcoma (bone formation). Changes in tumor phenotypic expression as aberrant as observed with the R3230AC line after *in vitro* passaging have not been reported. Apart from becoming an osteosarcoma, the tumor also became more metastatic. Cytogenetic analysis showed a decrease in chromosome number and the presence of a metacentric marker chromosome in later (>90) passaged cells.

The present study was undertaken to examine in detail the changes in phenotypic expression of the R3230AC line *in vivo* and associated

Accepted 8 June 1984.
*This research was supported by a grant from the National Cancer Institute of Canada.
‡To whom requests for reprints should be sent.

chromosomal changes after *in vitro* passaging. A correlation was made between bone formation as well as the increased metastatic ability and chromosomal changes of the R3230AC line.

MATERIALS AND METHODS

Animals, cell line and media

Female C. D. Fischer (F344) inbred rats (Charles River Breeding Laboratories Inc., Wilmington, MA), 8–12 weeks of age, were used in this investigation. The R3230AC mammary adenocarcinoma line syngeneic to F344 rats was obtained from Dr A. E. Bogden (Mason Research Institute, Worcester, MA). Growth characteristics and other properties of this tumor have been previously documented [14, 15]. Briefly, the R3230AC is a mammary tumor of spontaneous origin that has been serially transplanted *in vivo*. The tumor line consists predominantly of epithelial cells and a few stromal elements in which the histological organization resembles mammary gland alveoli [16]. The tumor line was adapted to grow *in vitro* cell culture. Cells were cultured in 75-cm^2 plastic flasks in RPMI medium 1640 containing 10% calf serum, penicillin G (100 units/ml) and streptomycin (50 μg/ml) (medium and added components were obtained from GIBCO, Burlington, Ontario, Canada). Cell cultures were grown at 37°C in a humidified atmosphere at 5% CO_2 and 95% air. Cells were subcultured every second to third day by the following procedure: old medium was removed, cells were trypsinized with 0.025% trypsin (Bacto Trypsin, Detroit, MI) for approximately 5 min, washed once and resuspended in new complete medium. Trypsin solution was made up in citrate saline pH 7.7. After 10–20 subculturings cells were frozen (liquid nitrogen) in complete medium plus 10% dimethyl sulfoxide (Sigma, St. Louis, MO).

Assay for lymphatic metastasis

A tumor footpad model was used to examine lymphatic metastasis to the draining popliteal lymph node [15]. Cells were injected subcutaneously in the right hind footpad and thereafter tumor size was measured using a dial gauge caliper (Schelltaster; H.C. Kroeplin, Hessen, F.R.G.). The left footpad was considered as normal and its width was subtracted from the width of the tumor-bearing footpad. Periodic palpation of the right popliteal lymph node was used to determine the presence of macrometastasis. At the termination of an experiment rats were killed and thoroughly examined for macrometastasis in the popliteal lymph node, lungs and other body organs. The animals were checked for micrometastasis by examining 5–8 histological sections of draining regional lymph nodes and lungs. The lymph nodes were fixed in alcohol–formalin–acetic acid solution, processed to paraffin wax, sectioned and then stained with hematoxylin and eosin. Other body tissues were processed and stained in the same way, except that Bouin's solution was used as a fixative.

Chromosome preparations

Cell cultures in log phase growth were treated with colcemid (CIBA) (0.06 μg/ml) for 2 hr at 37°C. The cells were then collected, centrifuged and resuspended in 5 ml of pre-warmed 0.075 M KCl solution for 10 min at 37°C. Treated cells were then fixed with two changes of methanol–acetic acid (3:1). Chromosome preparations were made using an air drying technique. Afterwards they were stained with Giemsa dye (Fisher Scientific Co. Giemsa stock, Sorenson buffer pH 6.8; 1:20) for 2 min, rinsed in distilled H_2O and air dried. About 30–40 metaphase cells were photographed and carefully analyzed. A trypsin technique was used to produce G-banding of chromosomes [17]. The G-banded chromosomes were classified according to the Committee for a Standardized Karyotype of *Rattus norvegicus* [18].

Electron microscope study

The R3230AC cells were prepared and examined under the electron microscope by Parsons' [19] method. Cells and spent cell culture medium (centrifuged) pellets were treated with a negative stain to examine for the presence of virus.

RESULTS

Tumor characteristics

The effects of *in vitro* passaging of R3230AC cells to their phenotypic expression *in vivo* were examined in detail. The histological identification of the tumor tissue examined has been based on the recent rat mammary tumor classification of Komitowski *et al.* [20].

At various passages, R3230AC cells grown in culture dishes were examined for cell type and shape with a microscope equipped with a micrometer. The cultures consisted basically of spindle and polygonal shaped cells. These cells were counted in three separate fields for each culture dish when cell growth was at a preconfluent stage. This allowed a general assessment of any major changes in the cell culture population. At passage 17 R3230AC cell culture consisted of spindle- and polygonal-shaped cells in relatively equal numbers (~1:1 ratio). When these cells were inoculated in the footpads of animals they formed primary tumors with a histology of well-differentiated adenocarcinoma consisting of many well-developed acini, and a high epithelial to connective tissue

ratio (Fig. 1A). The tumors grew very slowly and occasionally metastasized (~50% of tumor-bearing animals) to the draining popliteal lymph node (Table 1). Histology of the metastases was similar to that of the primary tumors, except for a higher connective tissue content (Fig. 1B).

The R3230AC cell cultures examined between passages 40 and 70 were found to have similar cell types as observed for early (17th) passaged cells. However, there was an increased amount of spindle-shaped cells as compared to polygonal-shaped cells (~2:1 ratio). Although there were no significant changes in growth or frequency of metastasis by cells from early passages, considerable changes in the histology of the primary and secondary tumors produced by these cells were observed. The histology of the primary tumor showed poor cellular organization, with many cells containing prominent pleomorphic nuclei. Cells of about the 66th passage when injected into animals produced tumors with a histology of a fibrosarcoma (Fig. 2). These tumors produced by cells of passages greater than 66 showed very little element of adenocarcinoma histology.

R3230AC cultures of passages greater than 90 consisted of mostly spindle-shaped cells with a much lesser number of polygonal-shaped cells (>5:1 ratio). It was observed that tumors produced by these cells had a faster growth rate than tumors from earlier passages (Table 1). The histology of tumors produced by cells passaged 90 times or greater showed bone development, which was in the form of intramembranous ossification without the presence of marrow. Tumors produced by these cells appeared as fibrosarcomas with occasionally small nodules of bone formation. At much higher cell passages bone formation in the tumors became more extensive and appeared at early stages of tumor growth. These tumors were identified as osteosarcomas (Fig. 3A); primary tumor tissue was poorly organized bone consisting of matrix and lacunae with osteocytes. The metastases of these primary tumors with bone development had a histology of an osteosarcoma (Fig. 3B). In all tumors produced by these later passaged cells acinar development was not observed. The tumors formed from cells of about passage 90 showed no change in metastatic ability. However, cells of passage 150 or greater produced metastases in 100% of the tumor-bearing animals.

A time course study was carried out to examine the events of tumor progression leading to bone formation. Five million R3230AC cells of passage 169 were inoculated into 12 animals. At 2-week intervals two animals were killed and primary tumors were examined. Tumors were palpable by 2–4 weeks post-tumor cell inoculation. At earlier stages of growth (after 2–4 weeks) tumors were identified as fibrosarcoma; poor tissue organization and abundance of fibrosarcoma was observed. After about 6 weeks of post-tumor cell inoculation bone formation was observed. At early stages of bone formation, small nodules of poorly organized intramembranous bone was found. As the tumor developed these nodules appeared to expand to eventually change the majority of the tumor tissue into bone.

Electron microscope study

Cells of passage 90 or greater were examined to determine if there was any contamination with cell-associated viruses. Electron microscope analysis of these cells and supernatants of the spent culture medium in which these cells grow showed no evidence of virus particles.

Table 1. *Characterization of the metastatic ability of the R3230AC cell line after* in vitro *passaging*

Cell passage No.	Latent period of tumor growth (days)*	Length of experiment (days)	Primary tumor diameter (mm), $x \pm$ S.E.†	Animals with metastasis‡
17	24	105	9.6 ± 0.4	(10)10/19
66	30	110	12.8 ± 0.6	(4)4/8
90	16	100	21.8 ± 1.6	(4)9/20
152	14	69	12.9 ± 0.4	(8)10/10

*Latency period before tumors began to grow. Values represent day (post-tumor cell injection) when primary tumor growth is observed in all animals.

†10^6 R3230AC cell were injected into the hind footpad. Measurement of primary tumor diameter in the footpad was at termination of experiment. Tumor growth of the various cell passages was linear overall.

‡Metastasis in tumor draining popliteal lymph node; No. of animals with metastasis detected in lymph node/No. of animals in experiment. Numbers in parentheses represent macrometastasis (palpable) in tumor draining lymph nodes at termination of experiment. Experiments were terminated when 100% of the animals exhibited metastasis or when the animals were cachectic.

Chromosome analysis

Chromosome analyses of R3230AC cells were carried out at various passages (Table 2). All the tumor cells analyzed from different passages were found to have a heteroploid chromosome number. In general, early passaged cells (21–43) were found to have a similar modal chromosome number. However, in later passaged cells (90–193) the modal chromosome number decreased; these cells had lost 5–7 chromosomes. A large metacentric marker chromosome (Fig. 4B) was found in all cells of passage 150 or greater, but only appeared in about 44% of the cells that had been passaged 90 times (Table 2). However, in earlier passaged cells (<90) this marker chromosome was not observed (Fig. 4A) after analyzing several hundred cells of different passages. The analyses of G-banding pattern of the metacentric marker chromosome revealed that it is an isochromosome formed by the long arm of chromosome No. 2 (Fig. 4C, D).

Table 2. Distribution of chromosome number in R3230AC cells after in vitro *passaging*

Passage No.	Range*	Modal No.*	% of cells with specific metacentric chromosome marker†
21	73–84	79	0
43	75–83	80	0
90	66–84	73	44
193	69–77	74	100

*Based on the analysis of 25 cells (photographs) or more of each passage.
†Based on the analysis of 200 cells of each passage.

DISCUSSION

To our knowledge this report is the first documentation of an aberrant change of a mammary adenocarcinoma line into an osteosarcoma by *in vitro* passaging. The changes *in vivo* involved several stages which were from a differentiated (glandular) to a poorly differentiated (fibrous) and then to a differentiated (bone) tissue. The increase in fibroblast-like cells (elongated spindle-shaped) in cell culture after many passages suggested that these cells could be responsible for the early sarcomatous transformation of the tumor cell line. The sarcomatous change of the R3230AC line does not appear to be due to epithelial cells. As to whether epithelial-like cells changed their morphology to fibroblast-like cells in culture is still debated [21]. Through continuous passaging, fibroblast-like cells may have been preferentially selected and become dominant in culture. These cells can survive longer and grow at a faster rate than epithelial-like cells in serially passage (*in vitro*) cultures. It has been reported that serial passaging of the mammary adenocarcinoma cultures can lead to the loss of epithelial-like cells and the predominance of fibroblast-like cells [4]. Furthermore, studies on mammary adenocarcinoma lines in culture have shown that when epithelial (polygonal) and fibroblast-like cells (spindle) are clonally selected and passage *in vitro*, they can produce adenocarcinoma and fibrosarcoma in inoculated rats respectively [4]. In late passages of the R3230AC cell line there was an increase of spindle-type cells in culture and a predominance of stromal elements in tumors produced by these cells. These results may be very similar to what other investigators working with mammary tumors have reported.

The relation between tumor progression and differentiation are not well understood. In the R3230AC system both these events are observed. Often found with serially transplanted tumors is the progression of the tumor to a more malignant and less differentiated state [7]. These changes may be the result of selection of phenotypically new clones or of pre-existing clones. In the R3230AC system the original tumor may have consisted of developmentally primitive-type (stem) cells. Through serial passaging these cells may have been selected and become dominant. Often found within tumors are abortive representations of differentiated cell type(s) and developmentally primitive cells [22–24]. It has been suggested that these primitive cells are responsible for rapid growth and maintenance of heterogenicity in tumors [24]. When these primitive cells are inoculated into a host they may then differentiate under appropriate conditions. This may be a possible explanation for the bone formation in the R3230AC system. Alternatively, the stromal cell components of the mammary adenocarcinoma may have been selected through *in vitro* passaging and become more malignant and anaplastic [4, 25].

The etiology of R3230AC tumor bone formation remains to be determined. Both DNA and RNA viruses can induce bone tumors [26]. On examination of later passaged R3230AC cells with the electron microscope, no evidence of virus particles was found. This suggests that bone formation is not due to a viral etiology.

Cytogenetic analyses were carried out to determine whether bone formation could be associated with cells having a specific chromosome pattern. Chromosome aberrations found in both animal and human tumors are considered as an integral part of tumor progression [27]. However, there has been no consistent correlation between metastatic potential of the tumors and their chromosome number or aberrations [27, 28].

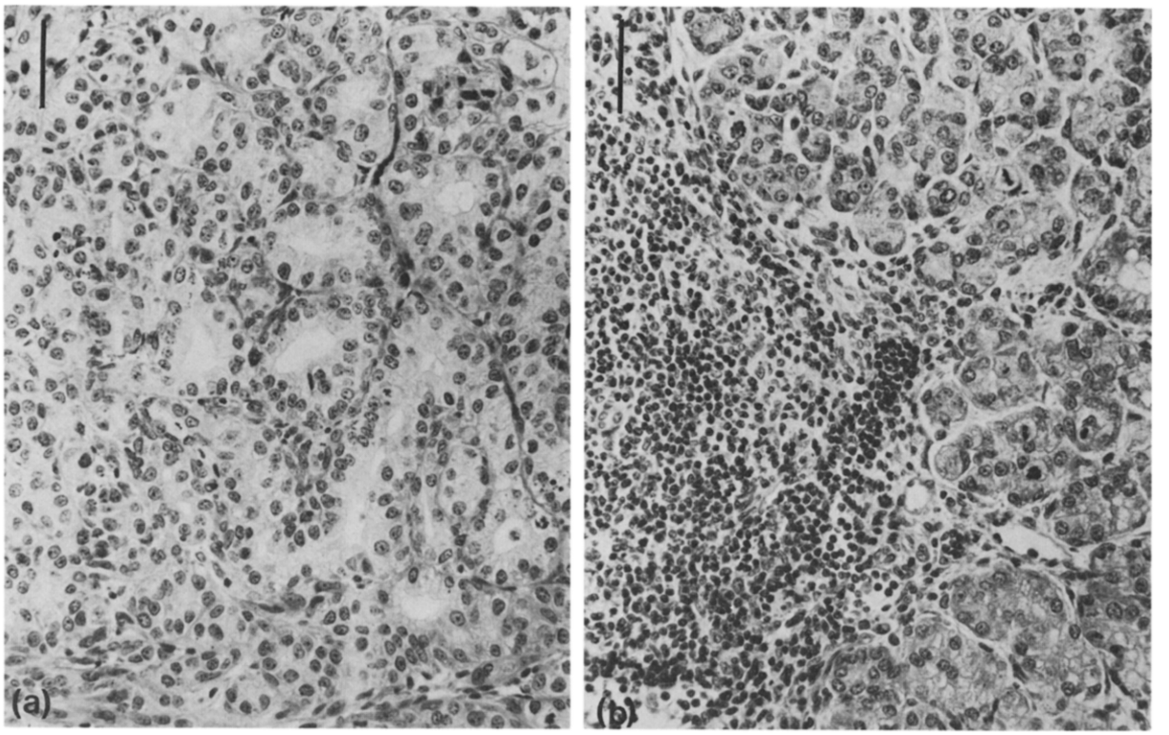

Fig. 1. *Histology of primary and secondary tumors of early passaged R3230AC cells. (A) Primary R3230AC tumor produced from cells of* in vitro *passage* 17. *Note well-developed acini. (B) Metastasis to the draining popliteal lymph node of the primary R3230AC tumor shown in (A). Note well-developed acini and tumor cell invasion into lymph node tissue. H & E. Bar* = 50 μm.

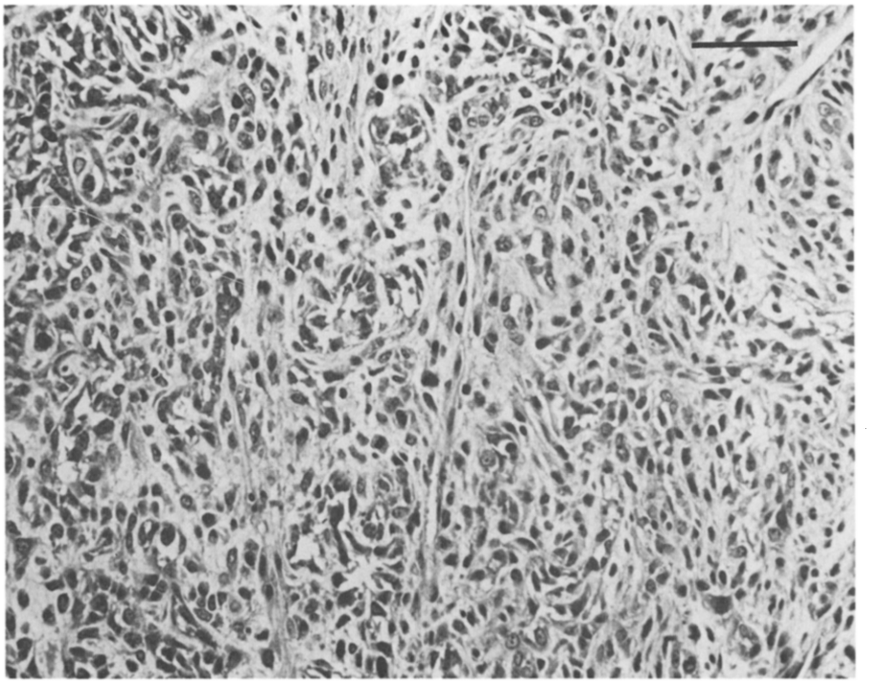

Fig. 2. Histology of primary tumor of intermediate passage of R3230AC cells. Primary tumor developed from cells of in vitro *passage 66. Note abundance of fibrous stroma and very poorly defined acini. H & E. Bar = 50 μm.*

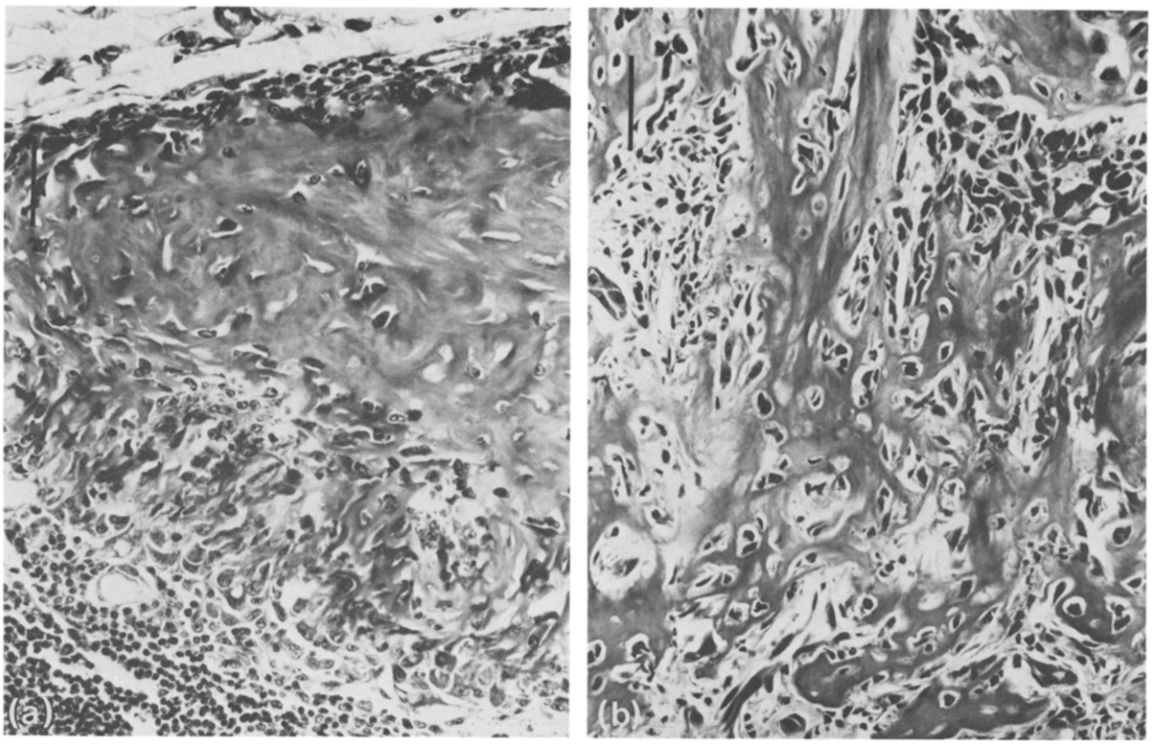

Fig. 3. Histology of primary and secondary tumors of later passaged R3230AC cells. (A) Primary R3230AC tumor developed from cells of in vitro *passage* 152. *Note primitive bone formation and fibrous tissue. (B) Metastasis to the draining popliteal lymph node of the primary tumor shown in (A). Note primitive bone formation and invasion into lymph node tissue. H & E. Bar* = 50 μm.

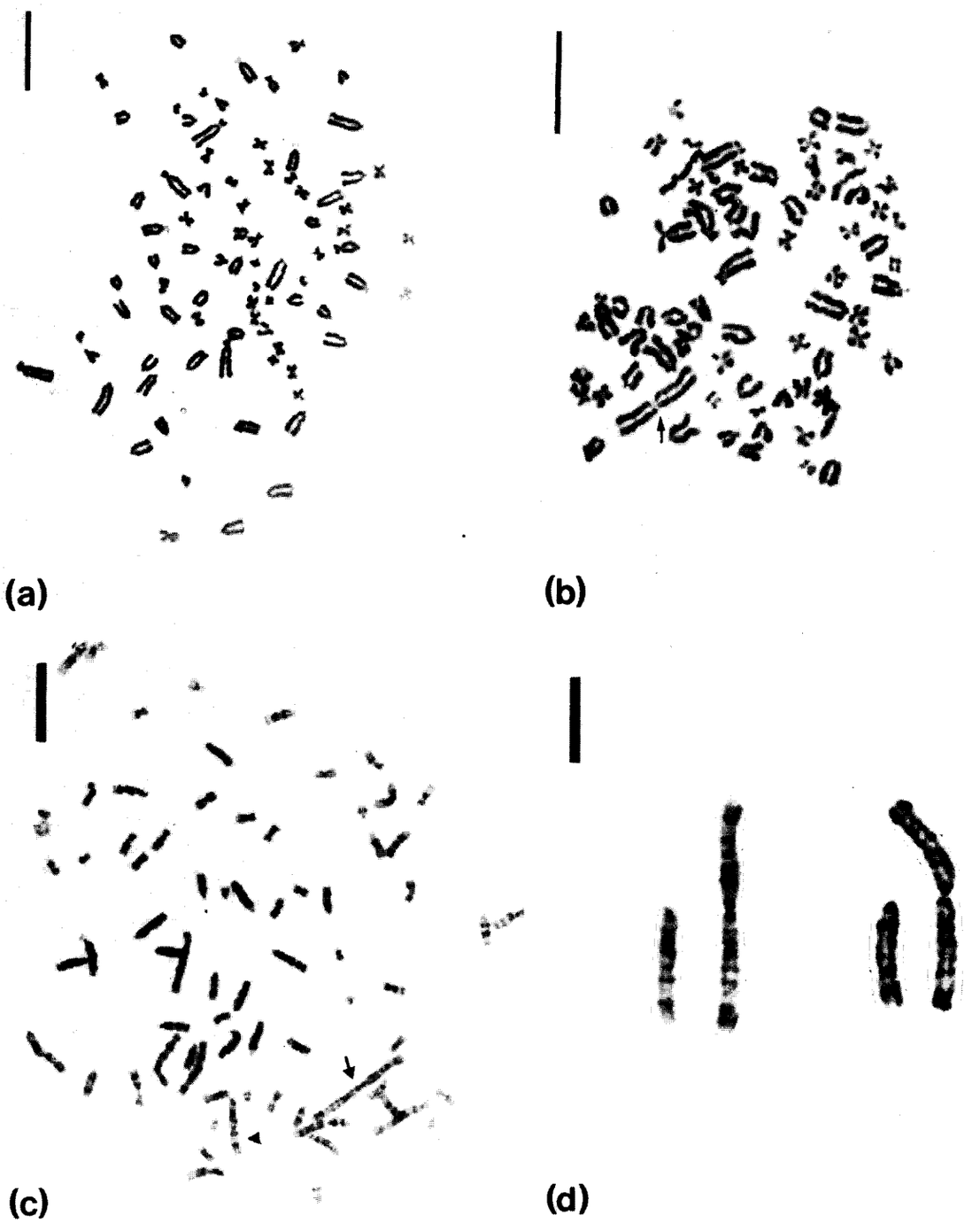

Fig. 4. Representative of heteroploid metaphases of R3230AC cells of different passages. (A) A representative of a heteroploid metaphase from R3230AC cells of passage 21. Bar = 10 μm. (B) A representative of a heteroploid metaphase from R3230AC cells of passage 190, containing a metacentric marker chromosome (arrow). Bar = 10 μm. (C) A representative of G-banding of a heteroploid metaphase from R3230AC cells of passage 190. The metacentric marker chromosome (arrow) is an isochromosome made of the long arm of chromosome No. 2 (arrowhead). Bar = 10 μm. (D) Two sets of G-banding chromosomes showing chromosome No. 2 (left side) and marker isochromosome (right side). Bar = 10 μm.

In later passaged (150 or greater) cells a specific metacentric marker chromosome was found, coincidentally of the same period when the R3230AC cell line showed a greater metastatic potential and the development of bone tissue.

The data obtained indicated that R3230AC cells containing this specific marker chromosome could be associated with bone formation or metastasis. The former, however, appears to be the most favorable. No marker chromosome was detected in early passage cells which were metastatic, and also, when the marker chromosome was found in 50% of cells of about passage 90 there was no increase in metastatic ability. Only in later passaged cells (150 or greater), when the marker chromosome was present in all cells, did the metastatic ability of the cells increase. Therefore the presence of this marker chromosome does not appear to directly correlate with the metastatic ability of the cells. Bone formation and the presence of the marker chromosome appear to correlate much better, i.e. when the marker chromosome appeared, bone formation was also observed. However, there is the possibility that the marker chromosome may not be related to either bone formation or metastasis.

The R3230AC cells containing this marker chromosome were most likely derived from a single cell which was then clonally selected during *in vitro* cultivation. The high degree of stability of this marker chromosome could be explained by the fact that the cells containing this marker have only one copy of normal chromosome No. 2 in every cell analyzed. Thus cells with a single copy of normal chromosome No. 2 cannot survive without the presence of this marker chromosome. It is of interest to note that a high frequency of trisomy No. 2 or presence of a marker chromosome involving chromosome No. 2 have been found in rat neoplasms [26]. In other tumor systems consistent associations have been found between tumor progression and genetic instability [7, 27]. This genetic instability coupled with clonal selection within the tumor has been suggested as the mechanism by which the tumor phenotype (histological) changes during tumor progression [7]. The presence of this marker chromosome may be useful for further investigations on R3230AC bone formation and metastasis.

Recently, emphasis has been placed on the development of experimental models of human osteosarcomas, particularly because of their devastating malignant characteristics. The present study can be related to the clinical situation since there have been clinical reports of osteosarcomas developing in the breast [28–30]. It has been suggested that human osteosarcomas of the breast can develop from mesenchymal tissue of fibroadenomas [29, 30]. The former tumors are also known to be rapid growing and highly metastatic [29]. In these clinical reports no drug or radiation treatments were implicated in inducing bone formation. The histogenesis of osteosarcoma development from breast tumors in humans is not fully understood. Further investigations on the phenomenon described in this report may shed light on the relation of tumor progression to tumor differentiation, in particular on histogenesis of bone tissue development in human breast tumors.

REFERENCES

1. Cairns J. The origin of human cancer. *Nature* 1981, **289**, 353–357.
2. Fould L. *Neoplastic Development*. London, Academic Press, 1969, Vol. 1.
3. Nowell PC. The clonal evolution of tumor cell populations. *Science* 1976, **194**, 23–28.
4. Malejk-Giganti D, Potter AH, Rydell RE. A fibrosarcoma component in culture of a chemically induced rat mammary adenocarcinoma. *Lab Invest* 1980, **42**, 627–635.
5. Horn H, Erlichman I, Geier A, Levij IS. Changes in morphology and hormone dependency following transplantation of rat 9,10-dimethyl-1,2-benzanthracene induced mammary adenocarcinoma. *Eur J Cancer* 1976, **12**, 189–194.
6. Bogden AE. Therapy in experimental breast cancer models. In: McGuire WL, ed. *Breast Cancer*. New York, Plenum, 1978, Vol. 2, 283–336.
7. Isaacs JT, Wake N, Coffey DS, Sandberg AA. Genetic instability coupled to clonal selection as a mechanism for tumor progression in the dunning R-3327 rat prostatic adenocarcinoma system. *Cancer Res* 1982, **42**, 2353–2361.
8. Al-Saadi A, Beierwaltes WH. Sequential cytogenetic changes in the evolution of transplanted thyroid tumors to metastatic carcinoma in the Fischer rat. *Cancer Res* 1967, **27**, 1831–1842.
9. Neri A, Nicolson GL. Phenotypic drift of metastatic and cell-surface properties of mammary adenocarcinoma cell clones during growth *in vitro*. *Int J Cancer* 1981, **28**, 731–738.
10. Mark J. Rous sarcoma in mice: the chromosomal progression during early *in vivo* transplantation. *Hereditas* 1970, **65**, 59–62.

11. Mitelman F. Comparative chromosome analysis of primary and metastatic Rous sarcomas in rats. *Herediats* 1972, **70,** 1–14.
12. Dzaelieva R, Schirrmacher V, Fusenig NF. Cytogenic changes during tumor progression towards invasion, metastasis and immune escape in the Eb/ESb model system. *Int J Cancer* 1982, **30,** 633–642.
13. Sandberg AA, Wake N. Chromosomal changes in primary and metastatic tumors and in lymphoma: their nonrandomness and significance. In: Arrighi FE, Rao PN, Subblefield E, eds. *Genes, Chromosomes and Neoplasia.* New York, Raven Press, 1981, 297–333.
14. Bogden AE, Kelton DE, Cobb WR, Gulkin TA, Johnson RK. Effect of serial passage in nude athymic mice on the growth characteristics and chemotherapy responsiveness of 13762 and R3230AC mammary tumor xenografts. *Cancer Res* 1978, **38,** 59–64.
15. Hoon DBS, Ziola B, Carlsen S, Warrington R, Ramshaw I. Circulating immune complexes and immunoglobulin M-class rheumatoid factor in rats bearing mammary adenocarcinomas which vary in ability to metastasize. *Cancer Res* 1983, **43,** 114–119.
16. Klein DM, Lozzi RF. Enhancement of R3230AC rat mammary tumor growth and cellular differentiation by dibutyryl cyclic adenosine monophosphate. *JNCI* 1977, **53,** 813–818.
17. Wang HC, Fedoroff S. Banding of human chromosomes treated with trypsin. *Nature* 1971, **235,** 52–54.
18. Committee for Standardized Karyotype of *Rattus norvegicus.* Standard karyotype of the Norway rat, *Rattus norvegicus. Cytogenet Cell Genet* 1973, **12,** 199–205.
19. Parsons DF. Negative staining of thinly spread cells and associated virus. *Cell Biol* 1963, **16,** 620–626.
20. Komitowski D, Sass B, Laub W. Rat mammary tumor classification notes on comparative aspects. *JNCI* 1982, **68,** 147–156.
21. Owen LN, Morgan DR, Bostock DE, Flemans RT. Tissue culture and transplantation studies on canine mammary carcinoma. *Eur J Cancer* 1977, **13,** 1445.
22. Das Gupta TK, Hajdu SI, Foote FW Jr. Extraosseous osteogenic sarcoma. *Ann Surg* 1968, **168,** 1011–1022.
23. Pierce GB, Shikes R, Fink LM, eds. *Cancer, a Problem of Developmental Biology.* New Jersey, Prentice-Hall, 1979, 1–83.
24. Mintz B, Fleischman RA. Teratocarcinomas and other neoplasms as developmental defects in gene expression. *Adv Cancer Res* 1981, **34,** 211–277.
25. Sanford KK, Dunn TB, Westfall BB, Covalesky AB, Dupree LT, Earle WR. Sarcomatous change and maintenance of differentiation in long-term cultures of mouse mammary adenocarcinoma. *JNCI* 1961, **26,** 1139–1157.
26. Finkel MP, Reilly CA, Biskis BO. Pathogenesis of radiation and virus induced bone tumors. In: Grundman E, ed. *Malignant Bone Tumors.* New York, Springer Verlag, 1976, 92.
27. Mitelman F. Tumor etiology and chromosome pattern-evidence from human and experimental neoplasms. In: Arrighi FE, Rao PN, Stubblefield E, eds. *Genes, Chromosomes and Neoplasia.* New York, Raven Press, 1981, 335–350.
28. Cifone MA, Kripke ML, Fidler IJ. Growth rate and chromosome number of tumor cell lines with different metastatic potential. *J Supramol Struct* 1979, **11,** 467–476.
29. Jernstrom P, Lindberg AL, Meland ON. Osteogenic sarcoma of the mammary gland. *Am J Clin Pathol* 1963, **40,** 521–526.
30. Benediktsdottir K, Lagerberg F, Lundell L, Thulin A. Osteogenic sarcoma of the breast. *Acta Pathol Microbiol (A) Scand,* 1980, **88,** 161–165.
31. Rottino A, Howley CP. Osteoid sarcoma of the breast: a complication of fibroadenoma. *Arch Pathol* 1945, **40,** 44–50.