Simplified Method for Establishment of Short Term Cell Lines from Human Mammary Epithelial Cells

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ABSTRACT: Most experimental and human cancers develop as a multistep process. Breast cancer too can be conceptualized to develop from a normal epithelial cell or its precursor, through multiple stages which are collectively known as benign proliferative breast diseases. This statement is supported by epidemiological proof, since as proliferative disease of breast goes through the stages of florid hyperplasia, atypical hyperplasia, and carcinoma in situ, there is correspondingly increasing risk of development of cancer. The epithelial lesion of "benign proliferative diseases" therefore can be considered to be borderline between normal and malignant cells and may provide significant information related to the process of carcinogenesis. Established breast cancer cell lines despite associated with certain disadvantages constitute an attractive in vitro model in breast cancer research. Non transformed cell lines of normal human breast and benign proliferative breast disease are not available and most of the established breast cancer cell lines too are derived from pleural effusions. Rodent models which had been used extensively earlier are now being discouraged due to ethical pressure on scientists to reduce or eliminate the use of animals in laboratory research demands to look for some other alternatives To cope with these problems this paper reports the establishment and characterization of short term (primary) cell lines from surgically excised and histolologically proven normal human breast, benign proliferative breast disease and breast cancer. These cell lines may prove an excellent model for understanding the various biological and molecular mechanisms of carcinogenesis.

Key Words - Breast Cancer, Primary cell Culture

I. INTRODUCTION

Breast cancer is the most common cancer and the second leading cause of cancer death among women in the West [1]. In India too it is the second most common malignancy in females, being second to cancer of cervix [2]. Therefore, it necessitates having an effective research model to understand the aetiology of breast cancer in order to attain effective therapy. Multiple experimental systems have been employed in attempts to elucidate the breast transformation process. Rodent models have been used most frequently [3]. In spite of its' contributions, this system has limitations. The cancers produced in rodents are not morphologically analogous to those in humans, and they do not invade and metastasize as do human cancers. The introduction of transgenic technology has added another dimension to the rodent model of breast carcinogenesis. Despite that considerable ethical pressure on scientists to reduce or eliminate the use of animals in laboratory research demands to look for some another alternatives.

Cell lines are widely used in many aspects of laboratory research and particularly as in vitro models in cancer research. They have a number of advantages; for example, they are easy to handle and represent an unlimited self-replicating source that can be grown in almost infinite quantities. In addition, they exhibit a relatively high degree of homogeneity and are easily replaced from frozen stocks if lost through contamination. However, there are disadvantages. Cell lines are prone to genotypic and phenotypic drift during their continual culture. This is particularly common in the more frequently used cell lines, especially those that have been deposited in cell banks for many years. Subpopulations may arise and cause phenotypic changes over time by the selection of specific; more rapidly growing clones within a population [4].Discrepancies in the most commonly, used breast cancer cell line, namely MCF-7, obtained from different laboratories had been reported [5]. As well as variations in cell growth rate, changes were observed in hormone receptor content, karyotype and clonogenicity, despite the cells appearing morphologically identical. Using 24-colour fluorescent in situ hybridization, this observation has again come to the fore, with MCF-7 cells from different UK laboratories showing markedly different karyotypes [6]. Additionally most of the established long term cell lines are derived from pleural effusions and not from primary cancer.

A viable alternative to using cell lines, either traditional or more recently derived, is to prepare primary cultures derived directly from tumours. This has a number of advantages [4]. Not only are cells directly isolated from the tumour site, but also detailed pathology is available to allow the characteristics of the culture to be compared with those of the original tumour. Broadly speaking, such cultures can be established either as explants, in which mixed cell populations grow out from small fragments of tissue. There are many benefits to be gained from using enriched primary cultures. In some cases, cells are only maintained in culture for a finite length of time, and they have little opportunity to undergo the transformations that are seen in the long-term culture of immortalized cell lines.

An important advancement in the knowledge of the pathogenesis of human breast cancer has come from the epidemiological and histopathological data of Page and Dupont [7].They demonstrated that the increasing risk for the development of cancer is proportional to the degree of proliferation (typical and atypical) in benign breast diseases. Thus epithelial lesion of the human breast can be conceptualized as a sequential spectrum, starting with non-proliferative diseases (including apocrine change, duct ectasia, and mild epithelial hyperplasia) leading to moderate or florid hyperplasia, atypical ductal and lobular hyperplasia, to ductal /lobular carcinoma in situ and invasive cancer.

This group of diseases can thus be utilised as a model system for understanding “carcinogenesis” by comparisons with normal cells on one hand and cancer cells on the other. Non transformed cell lines from normal and benign proliferative breast disease are not available with tissue culture banks. We report here the methodology for establishment and characterization of short term cell lines from histologically proven normal breast, benign proliferative breast diseases and breast carcinoma that may form an excellent model system for evaluating the pathobiology of breast cancer.

II. MATERIALS AND METHODS

2.1 Tissue collection and culture

Human mammary tissues were obtained from fresh biopsy specimen of patients with benign breast diseases and mastectomy specimens of the patients with cancer, prior obtaining their consent, from operation theatre (PGIMER, Chandigarh). Cell lines were obtained by a modification of the method of Hiratsuka et al [8]. The biopsy specimens were collected under aseptic conditions in cold Dulbecco`s modified Eagle`s medium (DMEM) supplemented with antibiotics (250 units/ml penicillin, 250 µg/ml streptomycin and 250µg/ml amphotericin B). After several washings with antibiotic supplemented medium, the extra adipose tissue was removed under a dissecting microscope. A portion of biopsy tissue was fixed for histological studies and adjacent tissues were finely chopped with care to avoid mechanical pressure on tissue. The minced tissue was subjected to enzymatic digestion using DMEM+7.5% fetal calf serum (FCS) + 10µg/ml insulin + 100 units/ml penicillin+250 µg/ml streptomycin and 2 mg/ml collagenase type IA in a 35 mm tissue culture petridish adding 4 ml of medium per petridish. The minced tissue was incubated at 37^0C in a humidified 95% air, 5% CO_2 atmosphere for 16- 18 hour.

The suspension was squirted several times to disperse the remaining fragments of tissue. The resulting mixture was filtered through a stainless steel cell dissociation sieve; mesh size No 40 (Sigma), to remove large connective tissue strands and non dispersed fragments. The filtrate was centrifuged at 120g for 10 minute. The supernatant was discarded. The pellet was resuspended in 10 ml growth medium (DMEM+10% FCS,10 µg/ml insulin, 20ng/ml EGF, 10 µg/ml apotransferrin,50ng/ml hydrocortisone,100 units/ml penicillin, and 250 µg/ml streptomycin) and left standing vertically for 15 minute. Nine millimeter of supernatant was removed. To the sediment, 5 ml of growth medium was added and allowed to stand for 10 min and the supernatant was again aspirated. The fourth sediment so obtained consisted of ductal/acinar epithelial cells devoid of their connective tissue sheath which was suspended in growth medium and plated into 25 cm^2 tissue culture flasks with 2ml growth medium per flask (Fig1). The cultures were maintained at 37 ^{0} C in a humidified 95% air, 5% CO_2 atmosphere. Twenty four hour later, 2 ml of growth medium was added. After 48 hour, the medium was changed to discard cell debris. The medium was changed at 3-4 day intervals. During this time radial outgrowths of epithelial cells from the organoids were observed

2.2 Subculture

The first subculture was done at an interval of approximately 10 days. The time of subculture was selected such that the epithelial colonies were large and no or minimal spindle shape cells were seen at the periphery of epithelial colonies. Subsequently, subcultures were done at near confluence (on an average of 7 days).The usual method of differential trypsinization was followed. The cells were washed twice with prewarmed Dulbecco Phosphate buffered saline to remove any traces of serum. This was followed by a rinse with 0.02% EDTA solution. The cultures were treated with 0.25% trypsin (cell culture grade, Sigma 3ml/25cm^2) for 5 -10 minute at room temperature. When the cells were sufficiently loosened, 5 ml of medium containing 20% cold FCS was added and the flask was gently agitated. A single cell suspension was obtained by gently squirting the medium with a tip of the pipette resting on

the bottom corner of the flask to avoid foaming. The dispersed cells were centrifuged for 5 minutes at 1000 rpm. A viable count was taken on a hemocytometer using Trypan Blue stain. The cell number was adjusted and seeded at 5 x 10^4cells/cm^2 into 25cm^2 tissue culture flask and 2x 10^5 cells into 35 mm tissue culture petridishes. An aliquot of cells from each batch were frozen in freezing medium (Medium +20% FCS+10%DMSO) so that cells can be retrieved when required. The third subculture cells were comprised of homogenous population of cells and were characterized for their epithelial cells

III. IDENTIFICATION OF CELL LINES

The cell lines grown in laboratory were identified as epithelial cell lines by phase contrast microscopy, immunocytochemistry and electron microscopy.

3.1 Phase Contrast Microscopy

Cultures were examined under an inverted phase contrastmcroscope for their morphology, growth characteristics, contamination and feeding

Immunocytochemistry

Indirect immunoperoxidase method was used on fixed cells[9]. The primary antibodies used were:

a) Monoclonal mouse anti-human epithelial membrane antigen (Dakopatts, dilution 1:100)
b) Monoclonal mouse anti-human pancytokeratin (Dakopatts, dilution 1:100)
c) Monoclonal mouse anti-human vimentin (Dakopatts, dilution 1:100)

Cultures grown on coverslips were washed with PBS three times, fixed in methanol for 30 minutes at room temperature and allowed to dry. Fixed cells were incubated with 200µl of primary antibody as per dilutions given above. Cells were washed three times with PBS and were incubated with a 1:50 dilution of peroxidase conjugated anti-mouse IgG for 45 minutes. Cells were again washed three times with PBS and the color was developed by diaminobenzidine tetra hydrochloride (0.5%) for 5 minute and counterstained with hematoxylin. A positive reaction was indicated by the presence of brown color.

3.2 Electron Microscopy

The cells were grown on culture coverslips (Costar). The coverslips, after being washed with PBS, were fixed in 2.5% gluteraldehyde, dehydrated and embedded in Epon. The covreslips were embedded on the surface of square molds with the monolayer facing down. After 24 hour of polymerization at 56^oC, the coverslips were peeled off, leaving the monolayer on the surface of Epon. A thin film of Epon was added and polymerization was continued for another 24-48 hour. Thin sections were cut at 65-70 nm and stained with uranyl acetate and lead citrate.

IV. RESULTS

Cell lines were established by the modified technique of Hiratsuka et al (1982), by changing the time period of enzymatic digestion (16-18 hr) depending upon cell type and histological confirmation of adjacent tissue from three distinct categories, as follows:

a) Four cell lines from histologically confirmed normal breast tissue designated HN-7, HN-10, HN-19 and HN-21
b) Four cell lines from benign proliferative breast disease showing moderate or florid hyperplasia BP-5, BP-17, BP-22 and BP-24
c) Two cell lines from malignant epithelial cells from carcinoma of breast BC-14 and BC-2

4.1 Morphological Studies

The epithelial colonies were visible after 5-8 days with cuboidal and polygonal outgrowths along with some elongated cells at the periphery from the organoids obtained in S_4 fraction in cultures from normal and proliferative diseases (Fig1).

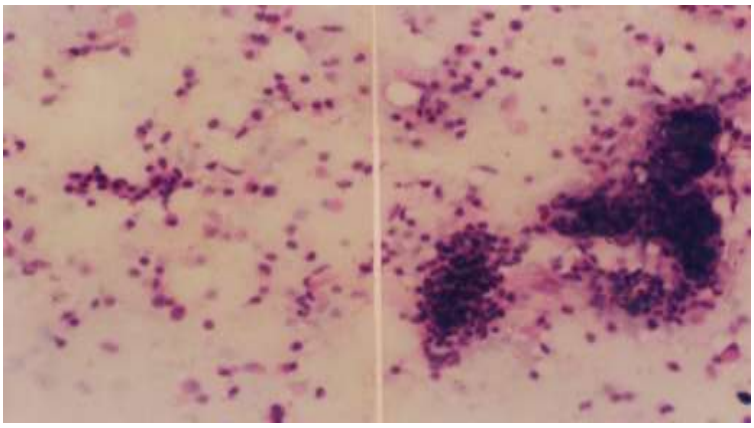

Fig.1 Hematoxylin and Eosin stanied smear of S1 fraction showing single cells or small clusters (Lt) and S4 fraction (Rt) showing aggregates or organoids of epithelial cells (X225).

When the primary cultures were 70% confluent with large epithelial colonies and no or minimal spindle shaped cells, subculture was done by differential trypsinization. Two cell lines from malignant epithelial cells from mastectomy specimen of carcinoma of breast were established. These cell lines were maintained in the same growth medium that was used for the establishment of cell lines from histologically normal breast and benign proliferative breast disease. Breast carcinoma cell lines formed continuous coherent sheets as compared to the colony formation seen in the cultures from histologically normal breast and benign proliferative breast disease. The primary breast cancer cultures grew slowly taking 2-3 weeks to become confluent and subculturing was done by the same method as mentioned above. The majority of the cells in confluent cultures maintained the round or polygonal shape.

4.2 Immunocytochemistry

Immunocytochemistry by the indirect immunoperoxidase method was done using monoclonal antibodies to cytokeratin and epithelial membrane antigen (EMA) as epithelial markers and vimentin as mesenchymal marker. Cytokeratin and EMA were strongly positive and vimentin was weakly positive in cells from histologically normal breast and proliferative benign breast disease. (Fig 2). In addition to EMA and cytokeratin breast cancer cells reacted strongly with caricnoembryonic antigen (CEA).

4.3 Ultrastructural Studies

The culture characteristics of the two groups of established cell lines from normal breast and benign proliferative breast disease were indistinguishable. The cells were generally round, polygonal or oblong in shape with round to oval vesicular nuclei and abundant cytoplasm. The nuclear chromatin was finely stippled and nuclei were prominent. In some areas well formed desmosomal junctions with bundles of tonofilaments converging to desmosomes and gap junctions were observed (Fig2). Electron microscopy analyses revealed ultrastructural similarities amongst the established human malignant breast cancer cell lines (BC-14, BC-25) and commercially available cell lines MCF-7 and T47D. Specialized junctional complexes, including desmosomes and occasionally finger-like projections of the cytoplasm in the intercellular space were observed. Intracellular lumina filled with microvillus projections and prominent surface microvilli typical of breast cancer were observed

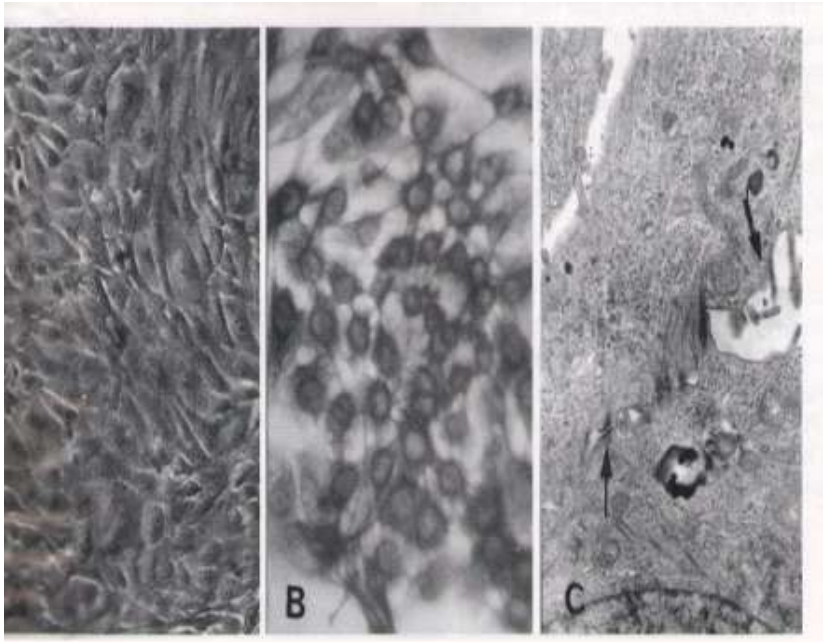

Fig.2: Identification of Cell lines. A. Phase Contrast photomicrograph of histologically normal breast epithelial cell line, showing polygonal cells with cobblestone appearnce (X 225). B.Immunoperoxidase staining of the same cell line showing intense cytokeratin positivity (x480). C. Electron micrograph of a cell line proliferative breast disease showing desmosomal cell junction (thin arrow) and microvilli (thick arrow) (X16,000)

V. DISCUSSION

A total of four cell lines from histologically confirmed normal breast tissues were grown successfully in laboratory by following the method of Hiratsuka 1982. Within 8 to 10 days primary culture became subconfluent which were subsequently passaged at an interval of 6 to 7 days. The epithelial nature of these cell lines was supported by their general morphological feature as well as ultrastructural features i.e. the ability to form colony, the formation of inter digitated cell contacts and well developed desomsomal junctions with intermediate filaments converging to the desomsone. In addition the cell showed high affinity to a panel of monoclonal antibody specific for epithelial cell. A strong positivity with keratin intermediate filament, characteristic of epithelial cells was observed in all the four cell lines [10] .The established cell lines also reacted with EMA, originally developed from milk fat globule membrane of mammary epithelial [11]. However, an apparent contradiction was noted since some cells were positive for the mesenchymal marker vimantin also. Similar co- expression of vimantin and keratin has been noted earlier in breast epithelial cells in culture [12,13] . In fact the expression of vimantin intermediate filament protein in culture is thought to dependent on the culture medium [14] and rate of proliferation. [15] or cell density [16]. Thus keratin positivity is considered as the more authentic marker of epithelial cells than vimantin positivity.

Similarly four cell lines from proliferative breast disease were established. Culture conditions were similar to that used for the cell lines from histologically normal breast with the variation in time period for the attainment of semiconfluency for the first passage (10 to 15 days) and subsequent passage (5 to 6 days). Immunostaining with EMA and cytokeratin confirmed the epithelial nature. Ultrastructural features were similar to those observed in histologically normal breast. With regard to the establishment of breast carcinoma cell lines, the isolation and establishment of long term cell lines from primary carcinomas has been relatively difficult due to technical problem associated with extraction of viable tumor cells from the neoplastic tissue or to the method of *in vitro* culture. Majority of breast carcinoma cell line which has been established so far have been obtained from secondary tumour and pleural or visceral effusion.

In this study the dissociative technique of Hiratsuka 1982 favoured the establishment of two cell lines from primary breast cancer. The cell line BC 14 was derived from patient with atypical medullary carcinoma whereas BC 25 cell line was obtained from comedo carcinoma. The primary culture grew slowly and became confluent in 2 to3 weeks for the first passage. A time period of 8 to 10 days was sufficient for the subsequent passage. Both cell lines displayed morphology consistent with the established cell line from primary human breast carcinomas [17]. Breast cancer cells formed monolayer of coherent sheets as compared to colony formation seen in other two histologically defined groups i.e. normal and benign proliferative breast diseases. The cells showed high affinity to panel of

monoclonal antibodies viz cytokeratin, EMA and carcinoembryonic antigen [18]. Ultra structurally in addition to exhibiting features confirming the epithelial nature of the cells, these cell lines showed a large number of surface microvillus projections, as well as multiple intracytoplasmic lumina filled with microvilli. The latter finding is a characteristic ultrastrcutural feature of human breast cancer and has been the experience of previous workers [19].

VI. CONCLUSION

Thus, to summarize, it may be conceptualized that benign proliferative breast diseases which pose an increased risk for the development of breast cancer in women form an intermediate step in the multistep process of development of cancer from normal mammary epithelial cells. This group of disease can thus be utilized as a model system for understanding "carcinogenesis", by comparison with normal cells on one hand and cancer cells on other hand. In this study non transformed epithelial cell line of histologically normal breast, benign proliferative breast disease and breast cancer have been established and characterized.

The results of this study indicate that human mammary epithelial cells from histological normal, benign proliferative breast diseases and breast cancer can be cultured as non-transformed cell lines. Primary cell cultures from above mentioned categories may form an excellent model to investigate various biological and molecular differences in the pathogenesis of breast cancer and in translational research in preclinical drug testing. These may be of use in predicting patients' response to chemotherapeutic agents.

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