

The Establishment of a Primary Colon Carcinoma Cell Line

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ABSTRAK

Titisan sel kanser kolon primer manusia (IMR 22) telah diperolehi di Institut Penyelidikan Perubatan, Kuala Lumpur daripada seorang pesakit berbangsa Cina berumur 71 tahun yang telah menjalani hemikolektomi kanan bag ketumbuhan di dalam sekum. Tumor tersebut telah dipastikan secara histopatologi sebagai 'well differentiated Adenocarcinoma Dukes Stage B'. Sekeping tisu tumor tersebut telah dipotong segar daripada bilik pembedahan dan dikulturkan untuk memperolehi titisan sel. Media pertumbuhan pemula, Amniomax C 100 telah disediakan dengan penambahar Fungizone, Penicillin, dan Streptomycin. Seterusnya ia digantikan kepada media 199 dengan garam Earl yang mengandungi 20% serum fetus lembu, Kanamycin, Penicillin, Streptomycin dan L-glutamina. Titisan sel IMR 22 telah diperolehi selepas sepuluh laluan dengan kehadiran sejenis sel di dalam kultur dimana ciri-ciri morfologinya menyamai dengan tumor primer.

Kata kunci: Titisan sel kanser kolon manusia, kultur tisu, histopatologi, media pertumbuhan, tumor primer.

ABSTRACT

A primary human colon carcinoma cell line (IMR22) was established in the Institute for Medical Research from a 71 years old Chinese man who had a right Hemicolectomy done for a fungating mass in the caecum. The tumour mass was then confirmed histopathologically to be a well differentiated Adenocarcinoma Dukes Stage B. A piece of the tumour tissue resected fresh from the operating theatre was grown to establish the cell line. The initial growth medium was in Amniomax C 100 supplemented with Fungizone, Penicillin and Streptomycin and was later changed to Medium 199 with Earl's salt supplemented with 20% fetal calf serum, Kanamycin, Penicillin and Streptomycin and L-glutamine. The IMR22 line was established after

ten passages with the occurrence of single type of cells in the culture whose morphological characteristics resembled that of the primary tumour.

Keywords: Human colon carcinoma cell line, tissue culture, histopathology, growth medium, primary tumor.

INTRODUCTION

Colorectal cancer, one of the most common cancers worldwide, is also one of the most frequently reported cancers in Malaysia (Ministry of Health, 2001). Colorectal cancer is a disease in which cells in the colon or rectum become abnormal and divide without control or order, forming a mass called a tumor. Colorectal cancer usually starts as a small polyp growing on the inside of the colon or rectum. Not all polyps turn into cancer, but removing these polyps early prevents them from turning cancerous. Polyps usually take between 5 and 15 years to become cancerous and things such as regular exercise may reduce the rate at which polyps grow (Kotasek & Pannall 1997). New approaches to systemic treatment for intestinal cancer in Malaysia are being sought. The approaches include the search for potential anti-tumour agents and the utilisation of combinations of drugs (chemotherapy) and radiation procedures (Rosenbaum 1983).

Cell culture can be used as a rapid, efficient, less expensive, better-defined and economical system in initial toxicity screening and in the elucidation of the mode of action of a drug (Dawson 1972). Cell cultures has also been widely used as a tool in many areas of basic biomedical research such as pharmacology, neurobiology, endocrinology, cellular physiology and studies on the biomedical basis of genetic diseases where the intact organ system is not possible to be employed. Lately, it has been the most commonly known system applied in cytotoxicity assays to search for novel and potential anti-cancer agents, antiviral assays, preliminary toxicity testing of industrial chemicals; in detection, quantification and study of mycotoxins and other food contaminants and in other *in vitro* assays of pharmacological agents (Shier 1991). As reproducible results are very important in any *in vitro* assay, the establishment of a permanent cell line has to be carried out. Later the cell line may be used in general studies such as regulation of mammalian cell proliferation *in vitro* or the development of *in vitro* toxicity assays. Tumour cell cultures were considered established when there was a single type of cell present in the culture, followed by several successful serial subcultures (Semple et al. 1978). In the present study, we aimed to isolate and establish a colon carcinoma cell line that resembled morphological and functional features expected of malignant cells.

MATERIALS AND METHODS

SAMPLE COLLECTION

A 71 years old man presented with a history of right abdominal distension and pain for a 6 month duration. At surgery, a fungating tumour in the ascending colon was noted covering more than 2/3 circumferences. A right hemicolectomy was performed and macroscopically showed a fungating mass in the caecum 9 cm from the distal margin.

CULTURING OF TUMOUR TISSUE

Two pieces of tumour tissue were received in separate sterile container, each containing 1 mm of tumour tissue taken from the fungating mass. The tumour tissue was sent to the Department of Histopathology, Universiti Kebangsaan Malaysia for routine histopathology. One piece of tumour tissue was analysed for DNA ploidy using a flow cytometer. The other was used to establish a cell line. The tumour specimen was minced up into small pieces and a few drops of Fungizone (Flow Lab, Australia) were added to reduce contamination. The tumour was divided into two parts, explant A and explant B. Explant A was transferred into a culture tube and 0.5 ml culture medium Amniomax C100 (Gibco Lab, UK) was added. Explant B was placed in a petri dish and 0.5 ml of collagenase was added. Both the explants were incubated for 24 hours in a humidified incubator at 37°C with 5% CO₂. At the end of the incubation, the tumour and supernatant of explant B were removed and centrifuged at 900 rpm for 5 min using KUBOTA 5100. The pellet was resuspended in 1 ml of culture medium and further incubated. The culture medium was changed every 4-5 days using the spinner spill-out technique until growth was observed (Semple et al. 1978).

TISSUE CULTURE

The initial growth medium was Amniomax C100 (Gibco Lab, UK) supplemented with fungizone (Flow Lab, Australia), Penicillin and Streptomycin (Flow Lab, Australia), T-flagyl and glutamine. After some growth was detected, the growth medium was then changed to Medium 199 with Earl's salt (Flow Lab, Australia) supplemented with 20 % fetal calf serum (Gibco Lab, UK), 50 mg/ml Kanamycin (Flow Lab, Australia), Penicillin and 100 IU/ml Streptomycin (Flow Lab, Australia) and L-glutamine. The supplemented culture medium was buffered with NaHCO₃ and N-(2-Hydroxyethyl) piperazine-N-(2-ethanesulfonic acid), pH of 7.

SUBPOPULATION

The cells were subcultured once a week using the spill-out spinner technique as described by Leibovitz (1975). The supernatant was then discarded and the cells were rinsed once with warm (37°C) Phosphate Bovine Saline (PBS) solution (per liter: NaCl, 8.0g; KCl, 0.2g; KH_2PO_4 , 0.12g; Na_2HPO_4 , 0.91g) pH 7.8. The cells were detached using 0.22 % (w/v) trypsin and then spin at 1000 rpm for 10 min. The pellet was resuspended into fresh medium and split two-fold into new culture vessels. Cells grown on slides were used for light microscopic studies. Hematoxylin, Dip Quick and Mucicarmin stains were performed according to Mallory (1961).

DETERMINATION OF GROWTH CHARACTERISTICS *IN VITRO*

Doubling time was determined from the cell growth curve. Six 25 cm² tissue culture flasks were seeded with 10⁵ cells in 5 ml of growth medium. The flasks were incubated at 37°C. Two cultures were taken after 24 hours incubation to establish the base-line count. The supernatant fluid was decanted and the cells were fixed for 5 min in absolute methanol, stained for 4 min in 1:21 Giemsa stained, rinsed in tap water and permitted to air dry. One hundred consecutive cell counts were made from each flask. The total counts in the two flasks were averaged and was divided by 100 to obtain the base-line count. Hayflick's formula was used to determine the generation time as follows (Hayflick & Moorhead 1961):

$$n = 3.32 (\log N - \log X_0),$$

in which n = number of generations, N = final population, and X_0 = initial population. Multiplication rate, r , or number of generations in a specified time, is calculated by:

$$r = n/(t_2 - t_0),$$

in which t_2 = number of time units of study (1 unit = 24 h) when N is determined, and t_0 = time unit at start of study (X_0 population or 1 unit). The generation time, g , or time for the population to double, is the reciprocal of r :

$$g = 1/r.$$

RESULTS AND DISCUSSION

The primary human colon carcinoma cell culture began to rapidly metabolize the culture medium within 48 hours after seeding. Fibroblasts were apparent initially in the culture and they disappeared after isolation during subsequent passages. The primary culture contained a single morphological variety of cell. Under phase-contrast microscope, the cells could be seen to be growing as closely packed rounded cells and in tightly packed clusters and would detach easily upon reaching confluence. The mass occupied the whole circumference and measured 6 X 3 cm. A histological section from the tumour showed a well differentiated adenocarcinoma Dukes stage B. Acinar structures were formed by the growth of the cells. The primary human colon carcinoma cell line (IMR22) was established after ten passages with the occurrence of single type of cells in the culture. The morphological similarity observed in this culture suggested the possibility of functional synchronisation. Mucicarmine stain indicated that all of the cells were mucin-producing cells as shown in Fig. 1.

The IMR22 showed the typical characteristics of any other cancer cell line *in vitro*. It was seen as epithelial-like sheets before the removal of collagenase and appeared as irregularly shaped clusters from fibroblastic areas after the removal (Figs. 2a and 2b). The presence of spindle-shaped fibroblasts in the culture, which may have arisen from the connective tissue, is common (Fig. 3). After a few passages, the cells grew in a disordered, helter skelter fashion, piling on top of each other until they produced a very crowded culture. The confluent cultures showed the "cobblestone pattern" morphology (Fig. 4). This pattern tends to become more fibroblast-like on repeated subculturing and can be avoided by subcloning and careful clone selection. The same features can be seen in the established colon carcinoma cell line, HT29 from the National Cancer Institute. The ability of the cells to form more than a single monolayer is actually the basis of the focus formation assay for oncogenic transformation. As the cells were attached less firmly to the substratum, they appeared to be more "rounded up" or boxy (Fig. 4).

In conclusion, the establishment of the colon carcinoma cell line IMR22 could be considered successful. However, further analysis and characterisation should be carried out to recognise its true status, as the isolation of cells from tumours may give rise to several different types of cell lines such as connective tissue fibroblasts, vascular endothelium, smooth muscle cells, infiltrating lymphocytes, granulocytes and macrophages and elements of the normal tissue which can also survive *in vitro*. The cultures originating from neoplastic tissues should meet several criteria such as the capacity to form a continuous cell line and the constituent cells normally are aneuploid, heteroploid, insensitive to density limitation of growth, less anchorage dependent and often tumorigenic (Preshney 2000). These

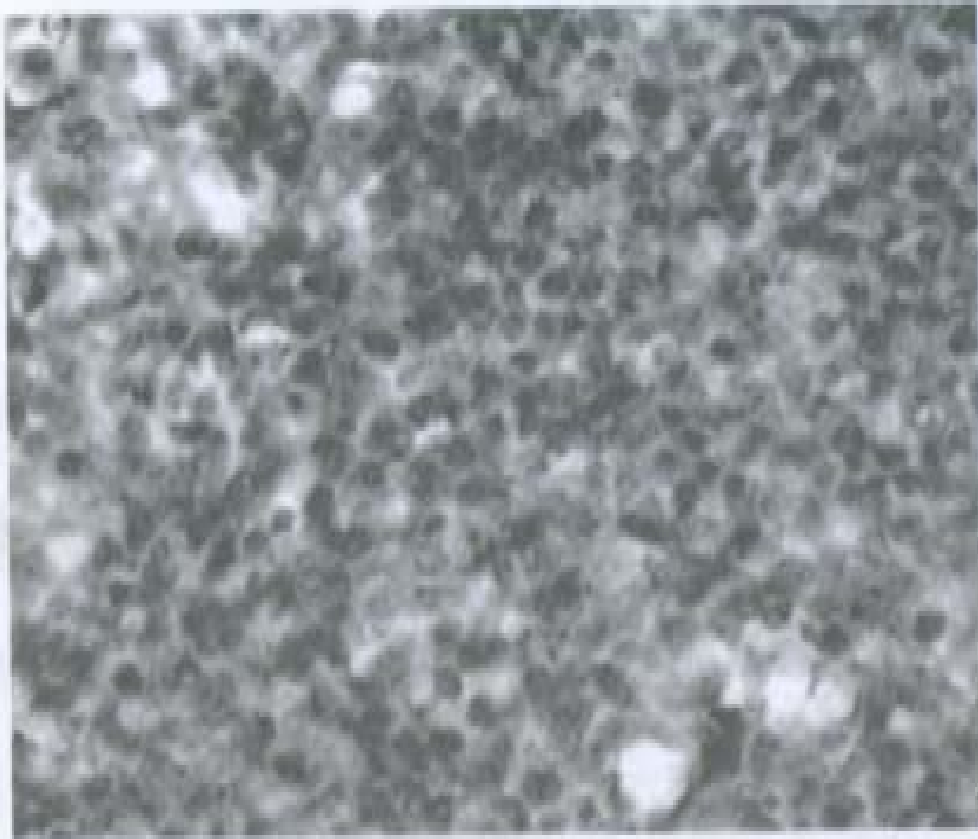


FIGURE 1. Mucicarmine stain showing all the cancer cells to be mucin producing (Photo magnification 20X)



FIGURE 2 (a). Cells and cell clusters from human colonic carcinoma after 48-hr dissociation in collagenase-before removal of collagenase (epithelial-like sheets)

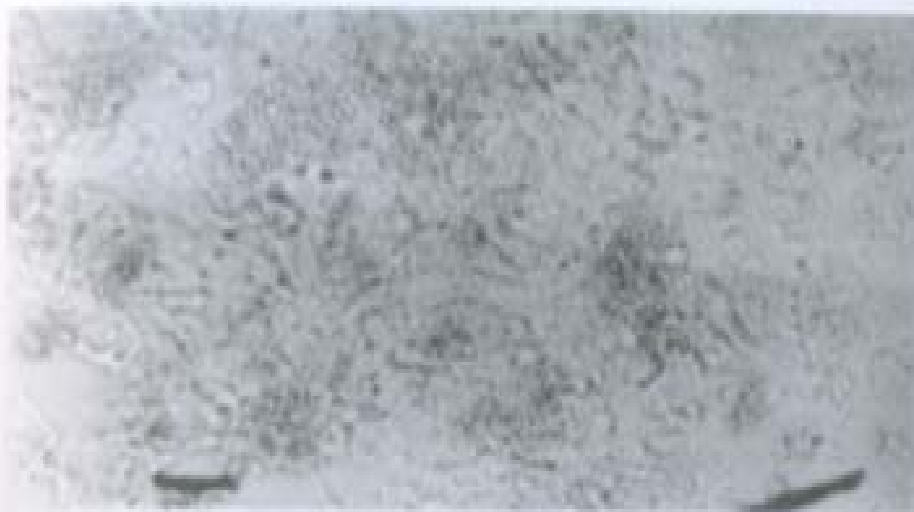


FIGURE 2 (b). Cells and cell clusters from human colonic carcinoma after 48-hr dissociation in collagenase-after removal of collagenase (more irregularly shaped clusters)



FIGURE 3. Early passages of cells. Note the occurrence of the spindle-shaped fibroblasts

characteristics will be tested in future to ascertain whether IMR22 demonstrates similar characteristics. Further investigations on IMR22 would include the development of unique markers such as specific chromosomal aberrations, enzymatic deficiencies and drug resistance.

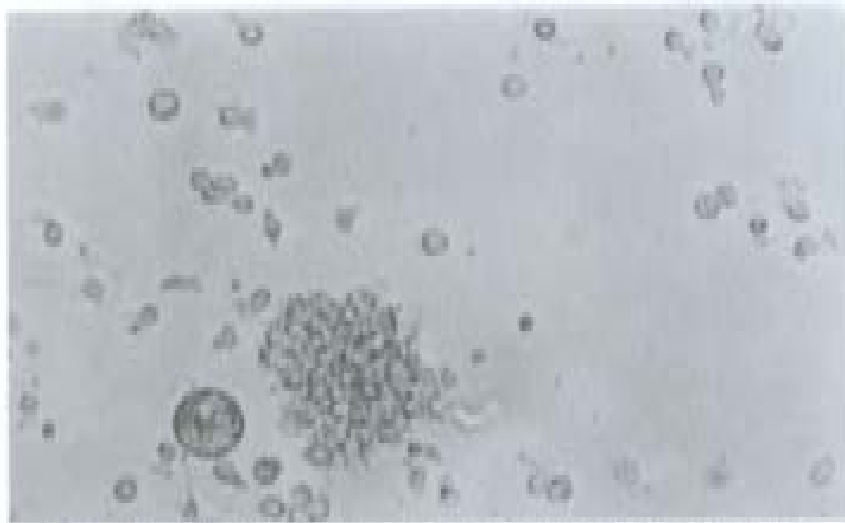


FIGURE 4. The third passages of cells. The cells clumped together forming a 'cobblestone pattern'. Note there were also single cells which were round in shape

ACKNOWLEDGEMENT

We would like to thank the Director of Institute for Medical Research for his permission to publish this article. This work was supported partially by the Intensification of Research in Priority Areas (grant number IRPA 94-62).

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