TISSUE CULTURE STUDIES ON NASOPHARYNGEAL CANCER

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Since the first successful attempts by Volpino (1) in 1910 to grow tumour cells *in vitro*, tissue culture studies have played an ever increasing role in the study of human cancer. Carcinoma of the nasopharynx, however, has been considerably neglected in the field of tissue culture, possibly due to the low incidence of this tumour in the main centres where this branch of experimental work is carried out. The purpose of this communication is to describe attempts in Singapore to grow the tumour *in vitro*, and to highlight the difficulties encountered to date.

METHODS

In all 14 cases have been examined, 10 of which were primary nasopharyngeal cancers and the remaining 4 were from metastatic deposits in cervical lymph nodes in proven cases. However in 5 of the cases from whom biopsies from the primary site had been obtained, treatment with radiotherapy had already commenced, and so they are being excluded from the present discussion. There was no selection in the cases examined, other than on the grounds of being clinically suspected as suffering from nasopharyngeal cancer. In each instance half of the biopsy specimen was examined histologically for confirmation of the diagnosis, while the other half was used for tissue culture purposes.

In a large proportion of cases the biopsy specimens, which were obtained as punch biopsies from the post-nasal space, were partially or wholly necrotic, and infected. The specimen was transferred to a sterile weighing bottle containing a small quantity of Hank's balanced salt solution, (to which the following antibiotics had been added; penicillin 100 units per ml., streptomycin 100 micrograms per ml., and "Mycostatin" 25 micrograms per ml.), and were taken to the laboratory.

Within ten minutes of receipt the specimen was washed in fresh Hank's solution prewarmed to 37 degrees centigrade, containing the above antibiotics in the previously mentioned concentrations, to free it from blood and purulent exudate. Only "fleshy" portions of the biopsy were selected, since the slimy necrotic remainder would certainly be non-viable.

Initially attempts were made to obtain a uniform cell suspension through enzymatic dispersion using an 0.25% solution of trypsin, (DIFCO 1:250), but because of the small quantity of suitable tissue available this method was abandoned.

The method later used was to dissect the tissue with minimum trauma, and small fragments measuring 1 mm. x 2 mm. were carefully transferred to a sterile 2 oz. prescription bottle using a Pasteur pipette. It was possible to arrange about eight to ten fragments in a 2 oz. bottle. The explants were allowed to "partially dry" by inverting the bottle in a hot-air incubator at 37 degrees centigrade for approximately twenty minutes before adding growth medium. An alternative procedure was to place the bottle in a refrigerator at 4 degrees centigrade, but results obtained by this method were less satisfactory.

The growth medium used throughout these experiments was Morgan, Morton, and Parker's medium No. 199, (2), supplemented with 0.5% lactalbumin hydrolysate, (NUTRITIONAL BIOCHEMICAL CORP.), and 15% calf serum, (MICRO-BIOLOGICAL ASSOC.). Penicillin and streptomycin in the previously cited concentrations were added to the Medium No. 199 by the manufacturers during preparation. "Mycostatin" was not routinely added to the growth medium, but was held in reserve and used only if fungal contamination occurred. During filtration with a Seitz filter carbon dioxide is lost, resulting in excessive alkalinity of the medium, and so carbon dioxide was introduced to re-adjust the pH to approximately 7.4.

Aliquots of one ml. of the completed growth medium were added cautiously to the prescription bottle to avoid detaching the explants, and the bottle was left undisturbed in the incubator at 37 degrees centigrade for at least forty-eight hours, after which they

were examined daily. Active cellular growth was shown by change in colour of the incorporated phenol red indicator from reddish-orange to yellow, and the growth medium was replenished accordingly. On average, change of growth medium was required only once or twice weekly. The best results were obtained by withdrawing only one-third to one-half of the old medium, and by adding an appropriate volume of fresh medium to bring the volume up to one ml. Samples of growth medium were withdrawn from the culture bottles at frequent intervals and submitted for bacteriological examination, to confirm freedom from P.P.L.O. and maintenance of sterility.

When cell multiplication had occurred to the extent that one-third to one-half of the total surface area available for cell growth was covered by a confluent cell sheet, subculture was carried out using a modification of Marcus, Cieciura, and Puck's technique, (3). This entailed using a bicarbonate buffered saline solution containing 0.05% trypsin and 0.025% versene (S.T.V. Mixture), to disperse the cell sheet. After removing the growth medium, 05. ml. of S.T.V. Mixture was added to the bottle and discarded after a few seconds to remove cell debris. 2.0 ml. of S.T.V. Mixture were then added and the bottle was incubated at 37 degrees centigrade for three minutes. An equal volume of completed growth medium containing serum was added to neutralise the effect of the trypsin, and the cell suspension was transferred to a small conical centrifuge tube and spun in a refrigerated centrifuge at 500 r.p.m. for five minutes. The supernatant was removed and the cells were resuspended in growth medium, and dispensed in one ml. aliquots into fresh sterile 2 oz. prescription bottles.

RESULTS

Using the method described above, it was possible to maintain cell growth and multiplication of explants of nasopharyngeal cancer *in vitro* for a maximum period of thirty-five days. For the initial forty-eight to seventy-two hours there was little or no activity, then outcrops of cells began to appear at the edge of the explants, as shown in Figure 1. These cells were round to polygonal in shape, with a nucleus which occupied one-half to two-thirds of the cell area, and their cytoplasm was clear and non-granular in so far as could be judged by examining unstained without phase-contrast microscopy. Within three to five days fusiform-shaped cells also appeared at the edge of the explants, and they soon assumed a characteristic spindle-shaped appearance as in Figure 2. These fibroblast-like cells were presumably derived from the connective tissue framework of the tumour. By approximately the end of the first week there was a mixed cell population consisting of these two cell types, and soon after this confluence began to occur.

In each of the five cases of explants from the primary site, a similar pattern of spontaneous degeneration occurred. The polygonal cells began to round off, and their cytoplasm which was previously clear now assumed a coarse granular character. The affected cells became progressively rounder and smaller until eventually they died and became detached from the glass, (see Figure 4). The fibroblast-like cells appeared to be less affected, and in each case they survived for a much longer period of time.

The average survival time of the cultures was thirty days, and although the time of onset of the degenerative changes varied slightly from case to case, the average time of onset occurred about the tenth day after explantation. Another phenomenon which was observed in each of the five cases was the formation of cell clumps, and this occurred just before or coincidental with the onset of the other degenerative changes described. These clumps continued to enlarge for a time and assumed a syncytial "honey-comb" appearance, before they began to decrease in size and disappear, (see Figures 2 & 3).

Attempts were made to grow the tumour *in vitro* from cervical lymph node metastases, using the method described above, but with a very limited degree of success. On each occasion there was exuberant fungal overgrowth, presumably occurring as a contaminant, since no fungi could be seen histologically in the tissue sections.

DISCUSSION

References to tissue culture studies on nasopharyngeal cancer are extremely scanty in the literature, and the only reference to work on this tumour to be found in the world literature was Ou's work which was reported

Fig. 1. Primary explant on 3rd day, showing outgrowth of polygonal cells. Magnification X 150. Unstained preparation.

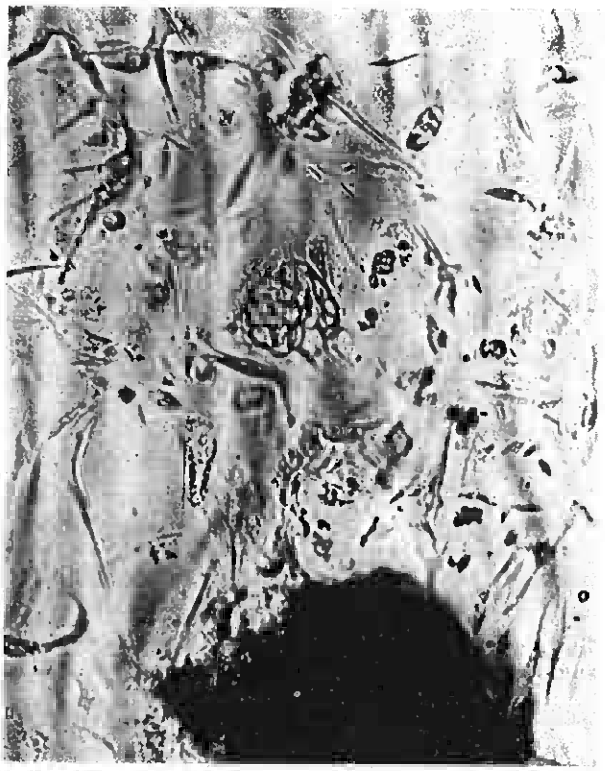

Fig. 3. Culture on 15th day, showing a typical "honey-comb" syncytium. Magnification X 150. Unstained preparation.

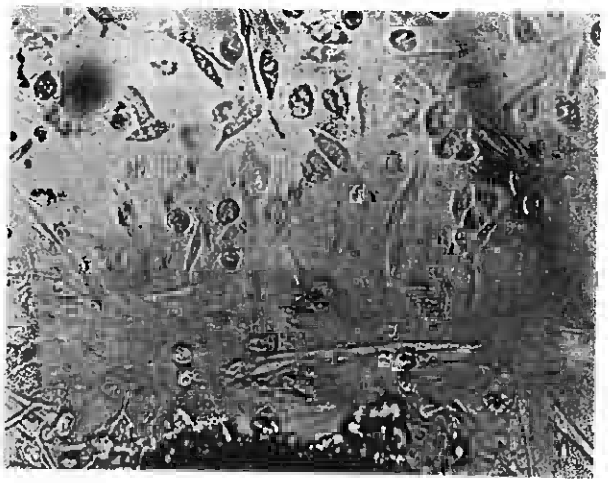

Fig. 2. Same on 7th day. Fusiform-shaped cells have appeared at the edge of the explant. An early stage of cell clump formation can be seen in the upper right corner of field. Magnification X 150. Unstained preparation.

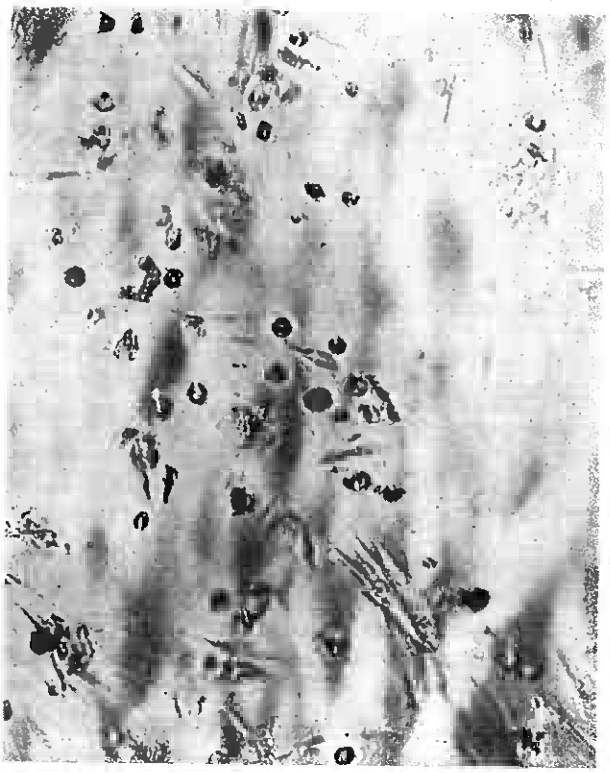

Fig. 4. Showing a late stage of degeneration. The epithelial cells have become rounded and pyknotic, and many have become detached. Fibroblast-like cells remain relatively unaffected. Magnification X 150. Unstained preparation.

at the 1964 Cancer Conference of the Chung Shan Medical College, and which is mentioned by Liang (4). Ou managed to maintain growth of nasopharyngeal cancer *in vitro* for a period of thirty-nine days, but the technique she employed, the nature of the growth medium used, and subsequent fate of the cultures were not stated.

Paul (5) in his monograph on tissue culture describes the KB cell line, which is one of the standard cell lines in use by virologists and workers in allied fields, as being derived from a nasopharyngeal cancer. Eagle (6), who originally isolated the KB cell line however, states that the cell line was derived from an epidermoid cancer of the floor of the mouth.

Because of the small size of the tissue fragments suitable for culture purposes it was not found feasible to obtain a uniform cell suspension through enzymatic dispersion with trypsin, although it is generally agreed that this is a more satisfactory method. By using the "feeder" technique devised by Puck and his associates (7,8), in which the cell suspension is introduced over a monolayer of previously irradiated cells which will continue to metabolise and so "condition" the growth medium, but which will not multiply, it may be possible to overcome the difficulty of a small initial inoculum of cells. In any work involving the possible isolation of viruses from the tumour tissue, the "feeder" technique would be open to the obvious criticism that the irradiated cells might be harbouring a latent virus infection, which would secondarily contaminate the nasopharyngeal cancer cells.

In considering the possible causes of spontaneous degeneration in tissue culture internal as well as external factors have to be taken into account. Although theoretically it should be possible to grow almost any viable tissue *in vitro* given adequate nutritional requirements and careful control of environmental conditions, in practice this is not true. Many tumours cannot be grown indefinitely *in vitro*, which may be due to some as yet unknown inherent factor in the tissue. Hayflick (9) has postulated a concept of senescence at cellular level as a possible cause of spontaneous degeneration in human diploid cells in culture.

The growth medium used throughout has been medium 199, which contains an almost complete supplement of amino acids and vitamins, and which has proved an adequate nutritional source for most tissue culture purposes.

The nutritional requirements of certain cell lines however are quite rigid, and unless the specific nutritional requirements are provided may result in failure of the cells or tissue to grow. Eagle and his associates (10) showed, for example, that although both HeLa and L cells required glutamine, this could be replaced by glutamic acid for HeLa cells but not for L cells.

Another cause of spontaneous degeneration in culture is the presence of a latent virus infection. It is interesting to note that Rowe and his collegues (1) noted spontaneous degeneration thirty-three out of fifty-three cases of primary explants of surgically excised human adenoids, an observation which ultimately led to his discovery of the adenoviruses. Spontaneous degeneration occurred over a period varying from eight to twenty-eight days.

SUMMARY

1. Using the technique of primary explantation, nasopharyngeal cancer has been grown *in vitro* using medium 199, supplemented with lactalbumin hydrolysate and calf serum, as growth medium.
2. The method employed is described.
3. The average survival time was thirty days, and in each of five cases there has been spontaneous degeneration followed by death.
4. The degenerative changes are described, and possible causes are discussed.

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