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A NEWLY ESTABLISHED CERVICAL CARCINOMA CELL LINE FROM A THAI PATIENT

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ABSTRACT

A cell named IST_3 was established from a squamous carcinoma of the uterine cervix from a Thai patient for use as a biological model for studying the effects of neutron radiation on the cell with and without boron compound. This project is part of Thailand's BNCT project. The doubling time of this cell line is 24 hours at passage 5. The cells have been grown well without interruption (January - July 1994) for 30 passages. Chromosome analysis at passage 14 showed numerical abnormality, 80% of the cells are polyploids, in a range of 71-98 with a modal number of 92. More than 40% of metaphases had somewhat similar two marker chromosomes which suspected to be derived by reciprocal translocation. Phase contrast microscopy revealed polygonal and pleomorphic in size and shape. Under the scanning electron microscope, the cells showed polygonal shape with numerous very long processes at the cell border. Interconnection between the tip of the processes of adjacent cells was observed. Transmission electron microscopy revealed desmosomes and tonofilaments of the cell thereby confirming its epithelial nature. Immunocytochemical staining of cytokeratin showed positive reaction of the epithelial cell type. Xenograft in nude mice is studied.

INTRODUCTION

Cancer is still a major health problem in developing countries as well as in Thailand. Because of its high incidence, cancer has been ranked as second to accident causing the country public health and economic problems and also loss of potency of life[(1)].

Carcinoma of the uterine cervix is the major cause of cancer death in Thai women today[(1)]. In the nationwide survey by the National Cancer Institute it has been found that carcinoma of cervix is the most prevalent cancer of all cancers affected in female in Thailand. It accounts for 13.9-18 % of all cases[(1)]. 87.5 % of total patients at Siriraj Hospital in Bangkok[(2)] and 76.69% of total patients at Chulalongkorn Hospital[(3)] with cancer of genital organ were found to have cervical carcinoma. From 1979 to 1983, the 1,364 patients with cancer of cervix were reported by Pathology Department, Faculty of Medicine, Chiang Mai

University[4]. In 1990 the Chiang Mai Maharaj University Hospital, admitted more than 600 patients with cancer in the cervix[5]. Most of these patients were in advanced stage and less than 35% can be cured by conventional irradiation, especially those in stage III B. The Boron Neutron Capture Therapy (BNCT) project in Thailand attempts to use the BNCT as a new therapeutic modality to treat the patients with advanced stage of cervical carcinoma. A number of cervical cancer cell lines having been established[6-8], including HeLa which has been reclassified as an adenocarcinoma of uncertain origin[9], have by now been extensively passaged in vitro. Despite the prevalence of the disease in Thailand there is no established cell line available for *in vitro* experiments.

Since 1993 the work of the project "Boron Neutron Capture Therapy" in phase II has been done by a multi-disciplinary research team in Thailand in cooperation with Uppsala University, Sweden and with assistance from the International Programme in Physical Science (IPPS). The project aims at "the establishment and evaluation of new techniques for treatment of uterine carcinoma by neutron from 252 Cf". The aims of the project in Neutron Radiobiology section, one part of the project, were to develop the primary cell culture of squamous cell carcinoma of the cervix from biopsy materials and to study the effects of boron neutron capture on cells by varying neutron doses with and without boron compound.

In early 1994 after we had done primary cell cultures from more than 30 biopsy materials of squamous cell carcinoma of the cervix, many groups of cells named IST1, ISTX, IST2 and IST3 were successfully established from a Thai Patient, aged 34 years old, diagnosed as cervical carcinoma of stage IIIB, keratinized and non-keratinized type. IST3 cells were chosen for a detailed biological study[10]. We describe here the establishment and characterization of a newly established cell line from human squamous carcinoma of the uterine cervix, for using as the in vitro model system for BNCT.

MATERIALS AND METHODS

Primary cell culture

A biopsy specimen was collected from a Thai patient suffered from a squamous carcinoma of the uterine cervix in the stage IIIB, aged 34 years old and was identified as mixed large cell keratinized and nonkeratinized type. The tissue biopsy was cut into small fragments (3 mm^3 in size) and washed three times with PBSA. The fragments were partially embedded into 25 cm^2 tissue culture flasks base-coated with a semisolid medium containing 0.4% agar (Bacto agar). Then RPMI 1640 supplemented with 10% fetal calf serum, 100 μg/mL streptomycin, 100 μg/mL kanamycin and 30 μg/mL ampicillin was added on the top of agar. Then the cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and maintained in a water jacketed incubator. After 1-2 weeks several single cells exfoliated from the biopsy specimens and then grew up as clustered floating cells. These cells were transferred into new tissue culture flasks containing 3 mL of 100% Chang medium and carefully changed the medium every week. After 3-4 weeks two small colonies consisted of 5-10 cells per colony attached onto the bottom of the flask were observed. Then we immediately washed out unattached cells three times with PBSA and leaved the attached cell grew continuously until the cells covered approximately 70% of the total area of the

culture flask. Then the first subculture was attempted. The tumour cells were washed with PBSA and then trypsinized with 0.25% trypsin (seromed^{R}) at 37 °C until all cells were detached from the culture flask. The action of trypsin was later stopped by adding the medium RPMI 1640 containing 10% fetal calf serum. These cells were collected and transferred into a new 25 cm^2 tissue culture flask and cultured in 3:7 mixture of Chang medium and RPMI 1640 containing 20% fetal calf serum. Subsequently, further subcultures were carried out when the cells in the flask were confluent and the cells were cultured in only the medium RPMI 1640 containing 20% fetal calf serum and antibiotics. The IST_3 cells grew well without interruption from January to July 1994 for 30 passages. Now they have been stored in liquid nitrogen in a growth medium containing 7% dimethyl sulfoxide.

Electron microscopic studies of IST_3 cells

For transmission electron microscopy (TEM), the cultured cells were collected by centrifugation at 4 °C, 1,000 rpm for 10 minutes. The cell pellets were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 at 4 °C for 1 hour. They were then rinsed several times with the same buffer and postfixed with 1% OsO4 in 0.1 M cacodylate buffer at 4 °C for another 1 hour. The fixed specimens were washed in cold distilled water and subsequently dehydrated in increasing concentrations of ethanol. At room temperature, the specimens were then infiltrated with propylene oxide, embedded in 2:1 and 1:2 mixtures of propylene oxide and Aradite 502 epoxy resin and pure Aradite resin respectively. Thin sections were stained sequentially with uranyle acetate and lead citrate and examined under a JEOL 1200 EX II transmission electron microscope at 80 kV.

For scanning electron microscopy (SEM) study, the cultured cells growing on coverslips were fixed in situ at 4 °C with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 and postfixed with 1% OsO_4 the same buffer. The fixed specimens were then rinsed, dehydrated and dried at a critical point. Following gold coating, the specimens were examined by a Hitachi S-430 scanning electron microscope at 25 kV.

Immunocytochemical study

In our immunocytochemical studies, anticytokeratin was detected by Streptavidin Biotin System using monoclonal antibody purchased from IMMUNON. Endogenous peroxidase was blocked by incubating the specimen in 0.1% hydrogen peroxide in water for 5 minutes. After washing several times, the specimens were incubated in the primary antibody for 30 minutes in a moist chamber and then in the ABC complexes. For visualization of the antigen-antibody complexes, hydrogen peroxide in water and 3-amino-9-ethylcarbozole (ABE) were premixed in N-N-dimethylformamide buffer (pH 5.2) and added to the coverslips for 15 minutes.

Chromosome analysis

The cancer cells at passage 14 were cultured for 72 hours and 100 μL of 10 μg colcemide/mL was added to the medium and continuously cultured for another 4 hours. The attached cells were removed and separated into single cells with 0.25% trypsin solution, centrifuge at 1,000 g for 5 minutes and treated with 5 mL hypotonic solution containing 0.075 M KCl for 10 minutes. They were then fixed, washed and suspended in ice-cold

glacial acetic acid : methanol (1:3). Cell smears were prepared by dropping a small drop of fixed cell suspension on slides, dried in a 60 °C hot air oven, and then banded chromosomes by using G-banding procedure. The chromosome number and the structural chromosome abnormality for a total of 310 spreads were analyzed.

RESULTS

Primary cell culture

We have been able to establish a cell line, designated as IST_3, from a cell culture derived from a Thai patient with a squamous carcinoma of the uterine cervix. IST_3 grew as adherent monolayers with characteristic epitheloidmorphology (Fig. 1a). The cells have been in culture for over 7 months (30 passages) and still retain their epitheloid morphology. A phase contrast micrograph of the culture in the 18th generation is shown in Fig. 1b. The cells were of polygonal and pleomorphic in size and shape. The growth rate of IST_3 cells was also studied and found that the cells have a population doubling time of 24 hours.

Electron microscopic studies of IST_3 cells

Under the scanning electron microscope, The IST_3 cells growing on coverslip showed numerous, very long, slender cytoplasmic processes on the cell surface (Fig. 2). Interconnection between the tip of the processes of adjacent cells was observed.

The transmission electron microscope observation has revealed the presence of tonofilaments (Fig. 3b and c) in the cytoplasm and numerous junctions resembling true desmosome between the adjacent cells (Fig. 3b) which is a feature that is typical to the epithelial cells *in vivo*. At the cell borders there are microvilli and interdigitated with microvilli of the adjacent cells (Fig. 3a).

Immunocytochemical study

From immunocytochemical staining, IST_3 cells were positive for cytokeratin expression which confirmed the epithelial origin of these tumour cells (Fig. 4)

Chromosome analysis

The chromosomal analysis of IST_3 cells at passage 14 showed a wide range in chromosome number from diploid through hyperdiploid to hypertetraploid. Eighty percent of cells were hyperdiploid with the chromosome number varied from 71 to 98 and the modal number was 92. Using of the G-banding technique we found two marker chromosomes in more than 40% of metaphases (Fig. 5). The banding pattern of these two chromosomes revealed that both were X chromosome but differed in length of the long arm. One had longer and another had shorter than normal. They were suspected resulting from the reciprocal translocation between the long arm of both marker chromosomes.

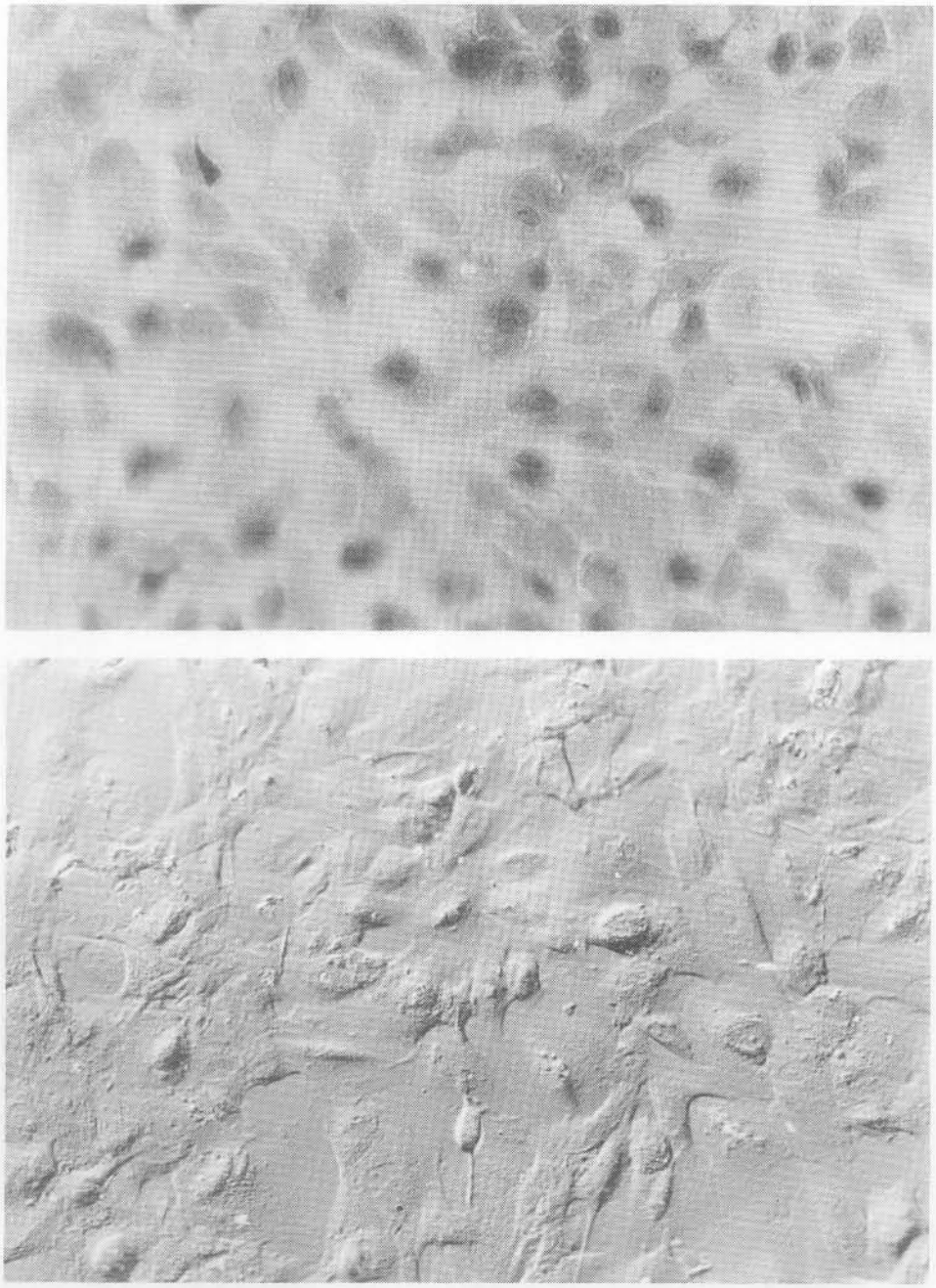

Fig 1. Polygonal and pleomorphic in size and shape of IST_3 cells (above x 200) with hematoxylin and eosin staining and (below x 200) with phase contrast microscopy.

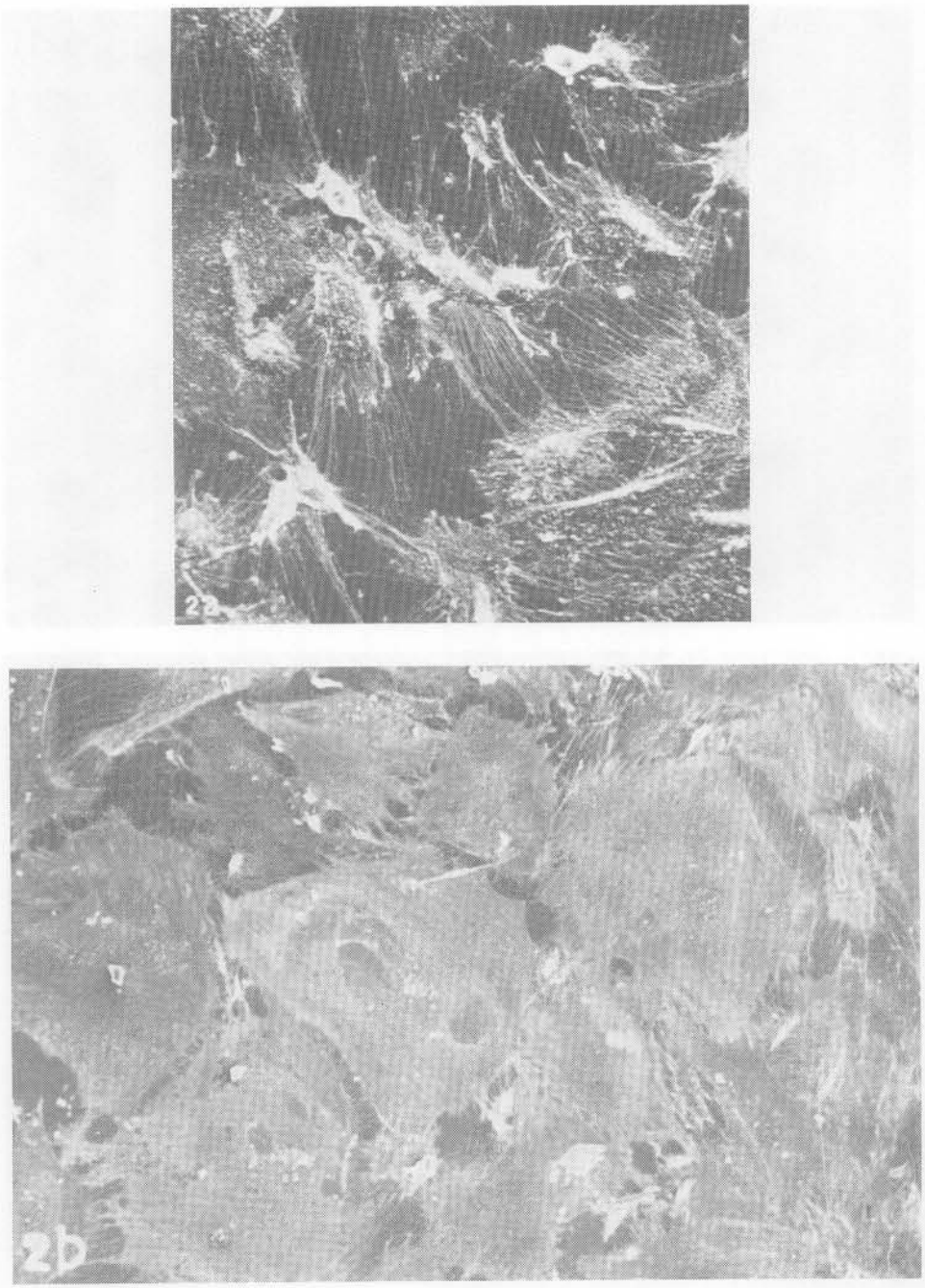

Fig. 2. Scanning electron micrograph of IST_3 cells growing on coverslip within loose area (a x 470) and dense area (b x 1020). Note that the cells are polygonal and pleomorphic in size and shape and numerous long cytoplasmic processes were observed.

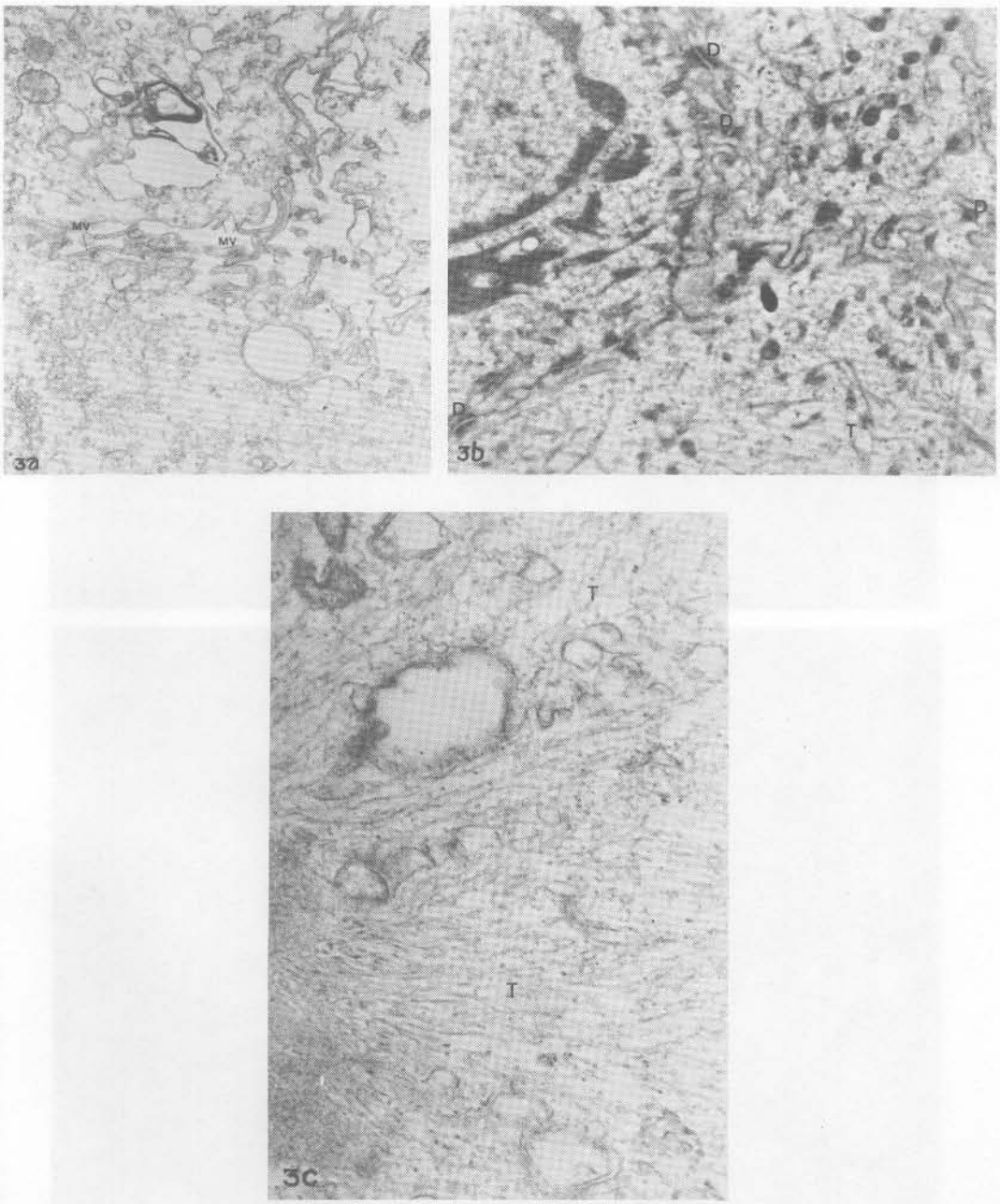

Fig. 3. Transmission electron micrograph of IST_3 cells. Microvilli (MV) are prominent at the cell borders (a x 6,270). Desmosomes (D) and tonofilaments (T) are present (b x 18,570), (c x 26,980).

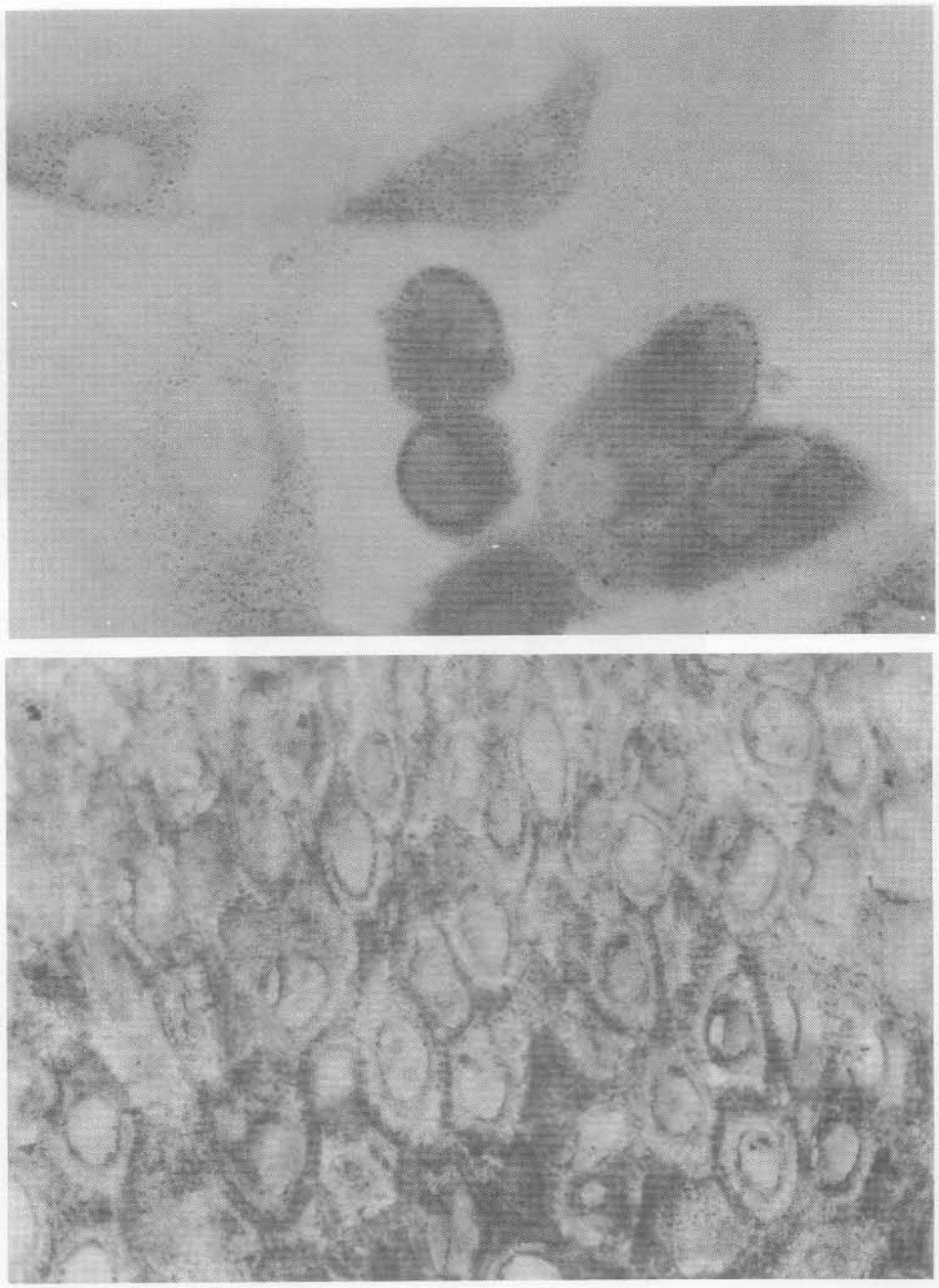

Fig. 4. Immunocytochemical staining of (above) IST_3 cells on coverslip and (below) the epidermal layer of human skin as the positive control with anticytokeratin antibody which specific for epithelial cell type. The positive reaction is shown as red dots in cytoplasm.

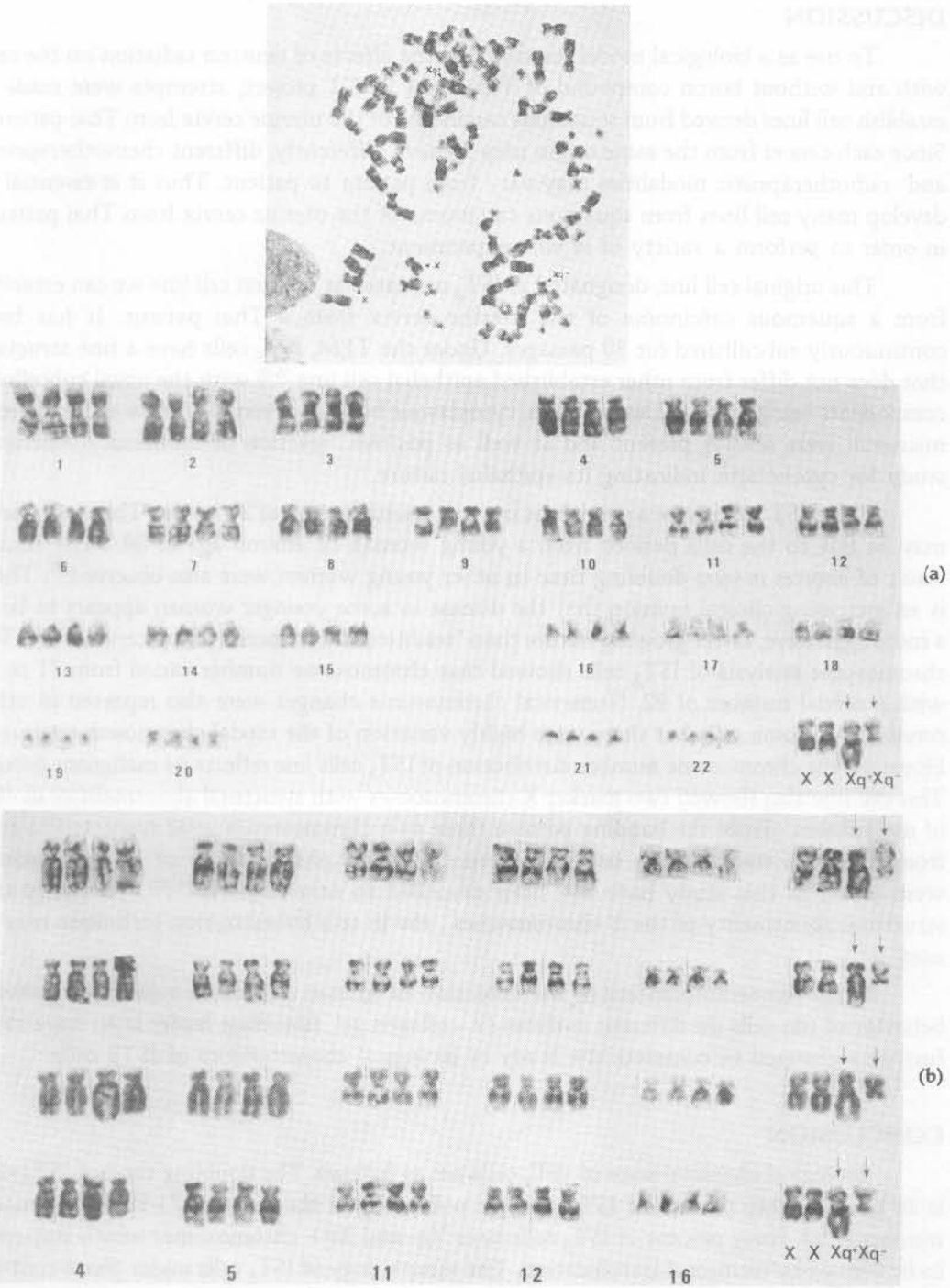

Fig. 5. G-banded karyotype of the IST_3 from (a) one cell and (b) four cells. All cells have a total number of 92 chromosomes. Structural abnormality of the two of X chromosome (arrows) which suspected resulting from reciprocal translocation are present.

DISCUSSION

To use as a biological model for studying the effects of neutron radiation on the cells with and without boron compound of Thailand's BNCT project, attempts were made to establish cell lines derived from squamous carcinoma of the uterine cervix from Thai patients. Since each cancer from the same origin may behave differently, different chemotherapeutic and radiotherapeutic modalities may vary from patient to patient. Thus it is essential to develop many cell lines from squamous carcinoma of the uterine cervix from Thai patients in order to perform a variety of *in vitro* experiment.

This original cell line, designated as IST_3 indicated as the first cell line we can establish from a squamous carcinoma of the uterine cervix from a Thai patient. It has been continuously subcultured for 30 passages. Under the TEM, IST_3 cells have a fine structure that does not differ from other established epithelial cell line [6-8] with the usual subcellular components being present. Desmosomes, cytoplasmic bundles of tonofilaments and numerous microvilli were always present and as well as positive reaction of immunocytochemical study for cytokeratin indicating its epithelial nature.

These IST_3 cells have a very short in vitro doubling time of 24 hours. This appearance may be due to the cells derived from a young woman of around age of 30. The similar result of shorter *in vitro* doubling time in other young women were also observed[7]. There is an increasing clinical opinion that the disease in some younger women appears to be of a more aggressive, faster growing nature than "traditional" carcinoma of the cervix [11-13]. The chromosome analysis of IST_3 cells showed that chromosome number varied from 71 to 98 with a modal number of 92. Numerical chromosome changes were also reported in other cervical carcinoma cells but there were highly variation of the modal chromosome number. However, the chromosome number distribution of IST_3 cells line reflects its malignant nature. This cell line also showed two marker X chromosomes with structural abnormalities in 40% of metaphases. From the banding pattern these two chromosomes were suspected to arise from reciprocal translocation between them. Structural abnormalities of X chromosomes were found in this study have not been described in other reports[14-15]. To clarify this structural abnormality of the X chromosomes , the in situ hybridization technique may be used.

In the xenotransplantation, the detection of human papilloma virus and growing behavior of the cells on different surfaces *i.e.* collagen gel, fibroblast feeder layer have to be further performed to complete the study of biological characteristics of IST3 cells.

CONCLUSION

Biological characteristics of IST_3 cells are as follows. The doubling time of IST_3 cells is 24 hours. Eighty percent of IST_3 cells are polypoids, in the range of 71-98 with a modal number of 92. Forty percent of IST_3 cells have Xq- and Xq+ chromosomes which suspected to be derived by reciprocal translocation. The morphology of IST_3 cells under phase contrast microscope is polygonal and pleomorphic in size and shape. Under scanning electron microscope IST_3 cells show polygonal shape with numerous very long processes at the cell border. Transmission electron microscope reveals desmosomes and tonofilaments of the

cells that confirm its epithelial nature. IST_3 cells show positive reaction with anticytokeratin antibody which confirms its epithelial nature.

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REFERENCES

1. Punthumchinda P and Sontipong S. (1989). *Thai Cancer Journal* **15**, 34-39.
2. Paiwuti S. (1989). Cancer of female genital organ. *Thai Cancer Journal* **15**, 135-145.
3. Vaivanijkul B. (1986). Cervical cancer at Chulalongkorn Hospital 1086. *Chulalongkorn Medical Bulletin* **30**, 839-848.
4. Theetranont C, Jiwiriyawat Y, Nteewattana S, Nimmanahaeminda K. (1985). Cervical carcinoma in north Thailand. *Chiang Mai Medical Bulletin* **2**, 135 145.
5. Statistical Report. (1990). Cancer unit, Faculty of Medicine, Chiang Mai University.
6. Chew EC, HO TH, Yeung SO, Hou HJ, Li RSC, Huang DP and Wong WS. (1990). Characteristics of a cell line established from a Chinese patient with a squamous carcinoma of the uterine cervix. *Anticancer Res* **10**, 213-218.
7. Kelland LR, Burgess L and Steel GG. (1987). Characterization of four new cell lines derived from human squamous carcinoma of the uterine cervix. *Cancer Res* **47**, 4947-4952.
8. Friedl F, Kimura I, Osato T and Ito Y. (1970). Studies on a new human cell line (SiHa) derived from carcinoma of the uterus I. Its establishment and morphology. *Proc Soc Exp Biol Med* **135**, 543-545.
9. Jones HW, McKusick VA, Harper PS and Wuu KD. (1971). "George Otto Gey." (1899-1970). The HeLa cell and re-appraisal of its origin. *Obstet Gynecol* **38**, 945-949.
10. Anusri Y, Phunchaisri B, Lorvidhaya V, Chaisukesant V, Lerprasertsuke N, Na Chiang Mai S, Petterson O, Mevatee U and Vilaithong T. (1994). A newly established cervical carcinoma cell line from a Thai patient. Abstracts. Int. Symp. on NCT for Cancer, Kobe, Japan, Oct. 31-Nov. 4, p 119.
11. Sidhu GS, Koss LG and Barber HRK.(1970). Relation of histological factors to the response of stage I epidermoid carcinoma of the cervix to surgical treatment. Obstet Gynecol 55, 329-338.
12. Stanhope CR, Smith JP, Wharton JT, Rutledge FM, Fletcher GH and Gallagher HS.(1980). Carcinoma of the cervix : the effect of age on survival. *Gynecol Oncol* **10**, 188-193.
13. Hall SW and Monaghan JM. (1983). Invasive carcinoma of the cervix in younger women. *Lancet* **2**, 731.
14. Herz F, Miller OJ, Miller DA, Auersperg N and Koss LG. (1977). Chromosome analysis and alkaline phosphatase of C41, a cell line of human cervical origin distinct from HeLa. *Cancer Res* **37**, 3209-3213 .
15. Atkin NB, Baker MC and Fox MF. (1990). Chromosome changes in 43 carcinomas of the cervix uteri. *Cancer Genet Cytogenet* **44**, 229-241.