Establishment and Characterization of a Tumor Cell Line from Human Nasopharyngeal Carcinoma Tissue

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ABSTRACT

An epithelial tumor cell line, CGI, was established from human nasopharyngeal carcinoma tissues. The CGI cells are of an epithelial origin as shown by their reactivities with the epithelial-specific antisera to keratin antibodies and by the presence of the desmosome structure at cell-cell junctions. CGI cells possess characteristics of tumor cells because these cells are tumorigenic in nude mice and also have reduced serum requirements for in vitro cultivation. The doubling time of CGI cells is 20 h and these cells have been successfully cultured in vitro for more than 200 generations. The average chromosome number of these cells is 60. Slot and Southern blot hybridizations showed the presence of Epstein-Barr virus DNA sequences in CGI cells. This cell line provides us an in vitro system for the study of the role of Epstein-Barr virus in nasopharyngeal carcinoma.

INTRODUCTION

NPC is a human squamous cell cancer which arises in the surface epithelium of the posterior nasopharynx. NPC can be classified into three types (I, II, and III) by the World Health Organization according to the degree of differentiation (1). Although the occurrence of NPC is rare in most parts of the world, this disease is common in certain regions of Southeastern Asia, Southeastern China, Taiwan (2, 3), East and North Africa (2, 4), and Alaska (5). Serological studies revealed that NPC patients have elevated antibody titers against the EBV-encoded capsid antigen and membrane antigen (6-8). Fahraeus et al. (9) recently examined NPC biopsies obtained from North Africa and China and found that EBV-encoded nuclear antigen 1 was present in 41 of 45 NPC tumor tissues. The latent membrane protein was also detected in 33 of 47 NPC biopsies. High levels of antibodies against the EBV-encoded DNA were also present in the sera of many NPC patients (10). Furthermore, DNA hybridization studies showed that EBV-DNA exists in all three types of NPC biopsy (2, 11, 12). Restriction fragments homologous to EBV sequences were also detected in NPC tissues passaged in nude mice (13). Northern blot analysis showed that EBV-RNA was also present in NPC tissue (14). Recent studies suggested that the pharyngeal epithelium expresses a functional EBV receptor (15, 16), and EBV is capable of infecting human epithelial cells in vitro (17). This evidence suggests that EBV is closely associated with NPC, even though the direct evidence of EBV being the etiological agent for NPC has yet to be established.

In this paper, we report the establishment of a NPC epithelial cell line. This cell line has unique biological properties which will provide important information for the study of the tumorigenicity of NPC.

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1 This work is supported by Chang-Gung Memorial Hospital Grant MRP 206.
2 To whom requests for reprints should be addressed.
3 The abbreviations used are: NPC, nasopharyngeal carcinoma; EBV, Epstein-Barr virus; FBS, fetal bovine serum.

MATERIALS AND METHODS

Establishment of Cell Line. NPC cell line was established from a biopsy obtained from a 47-year-old male patient with a WHO type II NPC tumor. This tumor has been diagnosed as a poorly differentiated squamous cell carcinoma. After removal of a tumor biopsy sample from the patient, the biopsy was immediately immersed in Ham's F-12 medium (Flow) and cultured according to the previously reported methods (18-20) with some modifications. The biopsy was minced into small tissue clusters less than 1 mm in diameter and washed several times in Ham's F-12 medium. The sample was then treated with pronase type XIII (Sigma) at a concentration of 200 µg/ml overnight at 4°C. The sample was broken into small pieces by pipetting. After 16 h, cells were harvested by centrifugation at 500 rpm. The pellet was resuspended in Ham's F-12 medium containing 10% FBS (GIBCO), 100 units/ml penicillin, 100 µg/ml streptomycin (GIBCO), and three growth factors: 10 µg/ml insulin (Sigma); 5 x 10^-4 M hydrocortisone (Sigma); and 25 ng/ml epidermal growth factor (Collaborative Research Inc.) (16, 17, 20). The resuspended cell clusters were then plated in Petri dishes precoated with FBS and incubated at 37°C in a 5% CO2 and water-saturated incubator. The medium was changed when most epithelial cells had attached. The culture was then propagated and passaged. During the first few passages, cells were incubated in 0.02% EDTA for 5 min. Fibroblasts were then removed by vigorous pipetting. Normally, epithelial cells remained adherent to the surface of Petri dishes. Subsequent culturing was carried out when cells reached 80% confluency in 100-mm tissue culture dishes. Growth factors were omitted from culture medium after the 10th passage (about 30 cell generations).

Detection of Cytokeratins and Desmosomes. Histological preparations were made from the tumor biopsy, cultured cells, and sections of nude mice tumors. These preparations were examined with the ABC method (Vector Laboratories). The cultured cells or frozen sections of tumor tissues were stained overnight at 4°C with polyclonal anti-keratin (Dako) antibody and with the monoclonal antibodies AE1-AE3 (Hybritech) which are specific for the human epidermal keratins. Antibody staining was visualized using a second-step biotinylated anti-mouse IgG and ABC reagents (avidin DH, biotinylated horseradish peroxidase).

For the detection of desmosomes, the confluent CGI cells were fixed in 4% glutaraldehyde, embedded in Spurr medium, and cut into 80-µm sections. The sections were stained in 5% osmium tetroxide and Sato solution and examined in a Jeol microscope (Japan). Serum Requirement. CGI cells (1 x 10^5) were plated in Ham's F-12 medium supplemented with FBS at concentrations of 0.625, 1.25, 2.5, 5, 10, and 20%. The cell number of the 48- and 96-h cultures was counted and the generation time was estimated.

Chromosome Analysis. The metaphase chromosomes in 100 cells were counted. The mid-log phase cells (about 60% confluency) were treated with Colcemid (Boehringer Mannheim) at a concentration of 0.2 mg/ml for 3 h. Cells were then scraped off from the dish and collected by centrifugation at 1000 rpm for 10 min. The pellet was then fixed with methanol:acetic acid (3:1) and stained in 5% Giemsa solution for 15 min. The chromosome number and aberrations were counted under microscope. One hundred metaphase spreads were counted for chromosome number and aberrations. The G-banding patterns of chromosomes in 100 cells were counted. The mid-log phase cells (about 60% confluency) were treated with Colcemid (Boehringer Mannheim) at a concentration of 0.2 mg/ml for 3 h. Cells were then scraped off from the dish and collected by centrifugation at 1000 rpm for 10 min. The pellet was then fixed with methanol:acetic acid (3:1) and stained in 5% Giemsa solution for 15 min. The chromosome number and aberrations were counted under microscope. One hundred metaphase spreads were counted for chromosome number and aberrations. The G-banding patterns of chromosomes were then determined by Seabright's method (21) with modifications. CG2 and CG3 cells were treated with trypsin at 12.5 µg/ml for 1 min and 30 s in Hanks' balanced salt solution (pH 8.0). The reaction was carried out in a 37°C water bath.

Tumorigenicity Study. To determine whether the CGI cells were tumorigenic, cells (1 x 10^5) of 5th, 10th, 21st, and 41st passages and
cells of different clones generated from CG1 cells, C2, C3, C5, and C12, were resuspended in phosphate-buffered saline and introduced s.c. into the ICR-nu/nu mice. The fibroblast cells derived from the same NPC biopsy were also tested in this study.

DNA-DNA Hybridization. High molecular weight DNA was extracted from the biopsy as well as from the different passages of CG1 cells following the procedure reported previously (22) with modifications. The tissue and cells were treated with lysis buffer containing 10 mM Tris (pH 8.0), 10 mM NaCl, 10 mM EDTA, 0.5% sodium dodecyl sulfate, and 1 mg/ml protease K (Sigma) overnight at 37°C. The DNA was extracted twice with phenol/chloroform isoamyl alcohol (49:49:2). The aqueous phase was treated with RNase A or RNase T1 for 1 h at 37°C before being extracted again with phenol/chloroform isoamyl alcohol. Finally, the DNA was precipitated with cold ethanol. The purified DNA was blotted onto nitrocellulose paper with a Manifold II slot blotter (S&S). A mixture of DNA containing 28 different electroeluted EBV BamHI fragments obtained from BamHI-digested recombinant plasmids (23) were nick-translated with [32P]-dCTP (24) and used as a probe for the detection of EBV-DNA in CG1 cells. The DNA-DNA hybridization procedure was reported before (25). The blot was prehybridized under conditions of 50% formamide, 5× Denhardt's reagent (with 0.02% each of Ficoll, polyvinylpyrrolidone, and bovine serum albumin), 5× standard saline-citrate (pH 7.0) and 200 µg/ml denatured salmon sperm DNA for 6 h at 42°C. The hybridization was carried out in 50% formamide, 2× Denhardt's reagent, and 1% sodium dodecyl sulfate with 1 × 10⁶ cpm/ml labeled probes at 42°C overnight. Five µg of CG1 DNA from different passages, P6, P18, and P30, were digested with BamHI, run onto a 0.7% agarose gel, and blotted onto a nitrocellulose membrane (26). The gel-purified EBV BamHI-K fragment was nick-translated and used as the probe for the Southern blot hybridization. The hybridization condition was the same as described above for the slot-blot hybridization.

RESULTS

In our studies, a total of 117 biopsies was obtained from the Department of Ear, Nose and Throat, outpatient clinics, Chang-Gung Memorial Hospital. Three of those biopsies propagated as primary cultures; only one of them grew unlimitedly and finally was established as a cell line (Fig. 1). This cell line, CG1, was derived from a WHO type II NPC tumor, a poorly differentiated squamous cell carcinoma. During the first few passages, cells grew in clusters and showed a typical boundary pattern (Fig. 1, A–C). These cells became more flattened after 10 passages (approximately 30 generations) (Fig. 1, D–F). CG1 cells were cultivated without a feeder layer and were maintained in the growth medium supplemented with only 5% FBS. The addition of growth factors such as insulin, hydrocortisone, and epidermal growth factor had no effect on cell growth. CG1 cells have been cultured in vitro for more than 1 year (at least 200 cell generations).

Keratin Analysis. Frozen sections of NPC biopsies and CG1 cells were examined with keratin-specific polyclonal antibodies or the monoclonal antibodies AE1–AE3 (27) for the detection of epithelial cells. Both polyclonal and monoclonal anti-keratin antibodies reacted with CG1 cells but did not react with a human primary fibroblast cells isolated from a NPC biopsy (Fig. 2) or B-lymphocytes (data not shown).

Desmosome Structure. The electron micrograph shows that CG1 cells can be easily identified as epithelial cells because of the intercellular connection of desmosome-like junctions (Fig. 3). Cytoplasmic ultrastructure was relatively simple but mitochondria were abundant. In addition, the microvilli were detected on the cell surface and intercellular spaces.

Doubling Time and Cell Growth. The doubling time of CG1 cells under optimal growth conditions (medium supplemented with 10% FBS) was about 20 h. However, similar to many other tumor cells, CG1 cells can also be cultured in low-serum media. The generation time was about 29 h in a medium supplemented with 2.5% FBS. The removal of the growth factors has no effect on the cell growth for the later passages (Fig. 4). In both 48- and 96-h cultures, the 5% FBS was sufficient for the cell growth while the serum concentration as low as 2.5% was enough for maintaining the growth.

The dividing cells were frequently observed to detach from the dish and then retouch the dish after the division which is a typical characteristic of tumor cells. The seeding efficiency was 80% at 4 h and 90% at 8 h after plating. The clonal growth can be obtained, but the split ratio of 1:3 maintains healthier appearing cells for routine passaging.

Chromosome Analysis. Chromosome irregularity (28, 29) including structural aberrations and abnormal chromosome number was observed in CG1 cells. The chromosome number was approximately 60 (Fig. 5). Ninety % of CG1 cells had a chromosome number ranging from 58 to 62. However, chromosomes of fibroblast cells obtained from the same NPC biopsy were normal (2n = 46). In earlier passages, severe chromosome abnormalities were often observed; more than 50% of the
chromosomes of the earlier passages were abnormal (Table 1). At P5, 54 of 100 cells showed either or both chromosome breaks and minutes. Chromosome breaks at centromere region were also observed. At P31, the degree of chromosome aberration decreased. Only 6 of 100 cells showed aberrations, among them 2 cells displayed both breaks and minutes and the translocation event still can be observed. The karyotype of CGI cells were nearly triploid with many marker chromosomes. The predominant karyotype was as follows: +5, +7, −8, +13, +17, +21, −Y, plus 11 abnormal marker chromosomes (Fig. 6). Marker chromosomes could be found in all cells examined with numbers ranging from 8 to 12. The origin of the markers have not been determined in the present study.

Tumorigenicity Study. CGI cells were able to cause tumors in nude mice. After the cells were introduced s.c. in these mice, 1-cm³ tumors appeared within 4 weeks. Fig. 7 shows the mouse given injections of CGI cells of P41 (about 120 cell generations) and a tumor was produced. Similar sizes of tumors were observed for P5, P10, and P21 and cell clones, C2, C3, C5, and C12. On the other hand, fibroblast cells derived from the same NPC biopsy did not form tumors in nude mice within 8 weeks.

Presence of EBV-DNA in CGI cells. Slot-blot hybridization analysis showed that DNA extracted from the biopsy and from CGI cells (P6, P16, P30, and P40) have homology with total EBV-DNA. Control experiments showed that two EBV-containing B lymphocyte cell lines, B85-8, and Namalwa, gave positive signals in the hybridization experiments, but a laryngeal carcinoma cell line, FADU (ATCC HTB 43), which does not contain EBV sequences gave a negative result (Fig. 8). Southern blot hybridization of BamHI-digested P6, P18, and P30 CGI cell DNA with a nick-translated EBV-BamHI-K fragment identified four discrete bands (6.8, 8.8, 13.5, and 17.5 kilobases, respectively) (Fig. 9). An EBV-negative cell line, CA46, gave no obvious hybridization signal with this probe. Two other EBV-positive control cells, Namalwa and CG3* showed a 5-kilobase band which was the same size as the probe (BamHI-K fragment from B95-8 strain).

DISCUSSION

In this paper, we have established a cell line, CGI, from a NPC biopsy. CGI cells can react with anti-keratin antibodies AE1-AE3. Only the cells with an epithelial origin were stained positive (shown as darker stain in cytoplasm). CGI cells were also routinely stained with AE1-AE3 to confirm their epithelial origin. Human primary fibroblast cells derived from a NPC patient were stained negatively with AE1-AE3.

**Fig. 2.** Immunostaining of frozen section of NPC biopsy and CGI cells. (A) The frozen section of the NPC biopsy was reacted with monoclonal anti-keratin antibodies AE1-AE3. Only the cells with an epithelial origin were stained positive (shown as darker stain in cytoplasm). (B) CGI cells were stained with polyclonal anti-keratin antibody. (C) CGI cells were also routinely stained with AE1-AE3 to confirm their epithelial origin. (D) Human primary fibroblast cells derived from a NPC patient were stained negatively with AE1-AE3.

**Fig. 3.** Electron micrograph of CGI cells. The desmosome (d) structure was observed in the cell-cell junction. Microvilli (mv) have been detected on the cell surface and intercellular spaces. × 12,000. n, nucleus.

**Fig. 4.** Serum requirement of CGI cells. The CGI cells (1 x 10⁴) were plated in the growth medium supplemented with FBS concentrations of 20, 10, 5, 2.5, 1.25, and 0.625%. The cell number of the 48-h and 96-h cultures was counted. •, cell numbers in the 48-h culture; ■, cell numbers in the 96-h culture. The data present are the average of 12 dishes for each serum concentration in three independent assays.

*Y.-S. Chang et al., submitted for publication.*
Fig. 5. Chromosome frequency distribution pattern. The chromosome number in 100 cells at metaphase was counted (19). The mid-log phase cells were treated with Colcemid at a concentration of 0.2 μg/ml for 3 h, fixed with methanol/acetic acid (3:1), and stained in 5% Giemsa solution for 15 min. Ninety % of the cells have a chromosome number ranging from 58 to 62.

Table 1 Chromosome aberrations of CGI cells

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<th>Passage no.</th>
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* Total CA, the sum of breaks, minutes, deletions, and translocations.

and contain the intercellular desmosome structure. This confirmed that CGI cells were of epithelial origin. Cells have also been cultured for more than 200 generations. Most tumor cells have been shown to secrete certain important growth factors (30-32); therefore they can be cultured in low-serum media. CGI cells have possessed this similar characteristic. Therefore, these cells are oncogenic because they have a low serum requirement and produce tumors in nude mice. We have also demonstrated that EBV-DNA sequences are present in CGI cells.

The establishment of carcinoma cell lines is often difficult. For example, two head and neck carcinoma cell lines were established from more than 100 surgical specimens (33). Because NPC biopsies are usually small and mixed with a large number of lymphoid cells, fibroblasts, and connective tissue, to establish a NPC cell line is even more difficult. We have tried 117 biopsies and succeeded with one.

The feeder cell system was widely used for the initial cultivation of epithelial cells (18, 19). However, the removal of feeder cells from the growing cells during the cocultivation becomes cumbersome. Therefore, we have avoided this method and chose a special growth condition which deprived the use of Feeder cells. Recently two epithelial cell lines were established with similar approaches (34).

Keratins are a group of water-insoluble proteins that form monofilaments in almost all epithelial tissues. The anti-keratin antibodies are useful for the positive identification of cells from an epithelial origin. Cross-sections of NPC biopsies were routinely examined with these antibodies to distinguish carcinoma from noncarcinoma. CGI cells were able to react with anti-keratin polyclonal and monoclonal antibodies indicating that they were of an epithelial origin.

The origin of CGI cells was further examined electron microscopically. The presence of desmosome structure was another indication for their being epithelial cells. The cytoplasmic ultrastructure showed abundant mitochondria and abnormal nuclei. Microvilli were detected at the cell surface and spaces between cells. The CGI cell DNA also contain human chromosomes (Fig. 6) and human alu sequences (data not shown). Therefore, we believe that the cells are of a human epithelial origin.

We have detected that EBV-DNA is present in NPC tumor cells. We used a mixture of 28 EBV-BamHI fragments which cover 99% of EBV genome as a probe to examine the CGI cellular DNA. We observed the positive hybridization signals with the NPC biopsy and cellular DNA of various passages of CGI cells. The electroeluted EBV-BamHI-K fragment from the
Fig. 7. Tumorigenicity test of CG1 cells. CG1 cells (1 x 10^6 CG1 cells) of the 41st passage (about 120 generations) were resuspended in phosphate-buffered saline and were introduced s.c. into the ICR nude mice. (A) Tumors 1 cm³ or larger appeared within 4 weeks. The tumors were surgically removed from nude mice. The sections of the nude mouse tumors were examined with hematoxylin and eosin staining and the chromosomes from the nude mice tumor cells were analyzed and proven to be of a human origin (data not shown). (B) The nude mice inoculated with fibroblast cells from the same NPC biopsy or phosphate-buffered saline alone gave no tumors within 8 weeks.

Fig. 8. Slot-blot hybridization of DNA from the biopsy and from the different passages of CG1 cells. High molecular weight DNA, starting with 2.5 µg and then a series of 2-fold dilutions, was applied to the nitrocellulose membrane. DNA from the biopsy, various passages, FADU, B95-8, and Namalwa cells were designated as Bi, P6, P16, P30, FA, B95, and Nal, respectively. Numerals at left, concentrations of DNA in µg.

Recombinant plasmid DNA hybridized with four species of CG1 DNA with molecular masses of 6.8, 8.8, 13.5, and 17.5 kilobases, respectively. The same pattern was maintained during the in vitro passages. Most interestingly, cells of those various passages preserved their tumorigenicity in nude mice. Cloning of EBV-DNA sequences in CG1 cells in a λ phage gt10 system allowed us to obtain four clones containing 5-, 6.8-, 8.8-, and 13.5-kilobases fragments, respectively (data not shown). The 5-kilobase fragment was not detectable with our Southern blot analysis but could be detected within the CG1 genomic library. Therefore, the presence of the 5-kilobase fragment in CG1 chromosome was the minor species compared to the other species of K-related fragments. The CG1 chromosomes have displayed dramatic abnormality during the passages (Table 1). Therefore, the other species, besides the 5-kilobase fragment, may be resulted from the chromosome exchanges and other abnormal activities during the in vitro passages. The further sequencing of those fragments was necessary for the understanding of the nature of the association between EBV-DNA and CG1 chromosomes.

The CG1 cell line will provide us an in vitro system with which to study the tumorigenicity of NPC at the molecular and cellular levels. Since the majority of the studies involving the expression of EBV genes have been limited to EBV-containing or EBV-immortalized B-lymphocytes (35), CG1 cells will provide us with an opportunity to study EBV gene expression in an epithelial tumor cell line. This will allow us to compare the EBV genes expressed in two different cell types (B-lymphocytes and epithelial cells) and possibly in two different cancers (NPC and Burkitt’s lymphoma) caused by the same virus. We believe that the establishment of this cell line will be important in helping us to understand the development of NPC and in providing us a system with which to study the interactions of EBV with their target cells.

In addition, radiotherapy and chemotherapy may alter the in...
vivo progression and cell heterogeneity of a tumor (36). The CG1 cell line was originally obtained from a patient who had never been treated with drugs or radiation at that time. We believe that such a cell line is more likely to represent the initial tumor than lines derived from therapeutically treated patients.

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