Establishment and Characterization of Human Fetal Liver Epithelial Cell Line Transfected with SV40 T Antigen (43900)

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Abstract. **The aim of the present study was to establish a stable cell line useful for the study of growth and differentiation of human fetal liver cells. We have established an immortalized human fetal liver cell line (designated NFL/T) by transfection with simian virus 40 (SV40) large T antigen, without any culture crisis. The cells showed growth properties similar to normal cells, including density-dependent cell growth. Electron microscopy demonstrated liver-specific differentiated morphological properties. Moreover, positive albumin and cytokeratin 7 production was detected immunologically. Thus, the NFL/T cell line is less transformed than the other fetal liver cell lines established by this method, and might be useful to study the growth and differentiation of human fetal liver cells.** [P.S.E.B.M. 1995, Vol 209]

The differentiation mechanism as well as the regulation of proliferation of human fetal liver epithelial cells is of particular interest. It has been postulated that both hepatocytes and biliary cells are differentiated from a uniform population of epithelial progenitor cells (1), for review see (2–4). Immunohistochemical studies have indicated that gradual phenotypic shift towards differentiated hepatocytes or biliary cells occurs during embryonic development (5–7).

Recently, various normal human cells have been immortalized by transfection with a plasmid vector carrying a simian virus 40 large T-antigen (SV40TAg) gene (8–14). However, human fetal liver cell lines which show evidence of differentiation, such as albumin expression, have not been established, although "stem cell"-like human fetal liver cell lines have been reported (14).

In this report, we describe the establishment of a new cell line, NFL/T, from human fetal liver after transfection with SV40TAg. The cells were not oncogenic and expressed some of the differentiated phenotypes. This NFL/T cell line would be useful for studying growth and differentiation of human liver cells in the fetal period.

Materials and Methods

Cell Culture and Transfection. Liver tissue (left lobe) was obtained from a normal female fetus aborted at the 18th week. Abortion was performed as medically indicated in the Nagoya University Hospital and informed consent was obtained beforehand. Cells were isolated by collagenase digestion of liver fragments and low speed centrifugation, and were cultured as a monolayer in collagen type I-coated plastic dishes as previously described (15). The cells were transfected by calcium-phosphate precipitation with 6 mg of the pMK16-SV40(ori$^-$) plasmid (16) (provided by Dr. T. Kunisada, Kumamoto University School of Medicine, Japan) for approximately 10,000 cells in a 60-mm dish. Incubation lasted 12 hr at 37°C in 2 ml of transfection medium. The resultant cell line, NFL/T, was cultured in collagen type I-coated dishes using Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). The medium was replaced every 3 days.

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Electron Microscopy. Pelletted cells were first fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 *M* phosphate buffer (pH 7.4) at 4°C for 2 days. They were postfixed with 1% osmium tetroxide, dehydrated and embedded in resin. Ultrathin sections were electron-stained and observed with a Hitachi H-800 electron microscope.

Cell Growth Assay and Proliferation Analysis. For cell growth assay, 4.0×10^5 cells and 1.6×10^4 cells were plated in 60-mm plastic culture dishes coated with collagen type I-dishes. Cells were counted using a hemocytometer.

DNA synthesis, measured by 5-bromo-2'-deoxyuridine (BrdU) incorporation, was detected immunocytochemically with Amersham's cell proliferation kit (Amersham, Buckinghamshire, United Kingdom). Briefly, cells on a glass coverslip were incubated for 24 hr at 37°C with labeling medium, which was prepared by diluting BrdU labeling reagent (RPN201) at 1:1,000 with MEM/10% FBS. The cells were then fixed in acid-ethanol solution (95% ethanol, 5% acetic acid). After the cells were incubated with anti-BrdU monoclonal antibody, the bound antibody was detected using peroxidase conjugated antibody to mouse immunoglobulin and polymerizing diaminobenzidine (DAB) in the presence of cobalt and nickel, giving blue-black staining at BrdU incorporated nuclei.

Tumorigenicity. Athymic nude mice (BALB/c *nu/nu*) were injected subcutaneously with 200 μl of a cell suspension (containing approximately 6×10^5 cells) at each of two injection sites in the subscapular region. Animals were monitored over a 10-month period for tumor formation at Institute for Laboratory Animal Research, Nagoya University School of Medicine.

Indirect Immunofluorescence. Cells were grown on Chamber-Tek chamber slides (Miles, Naperville, IL) and fixed in absolute ethanol. They were incubated with mouse monoclonal antibodies to the following proteins: large T antigen (Oncogene Science, Manhasset, NY); human cytokeratin 7 (Cymbus Bioscience, Southamption Hampshire, United Kingdom); human cytokeratins 8, 18, and 19 (Cymbus Bioscience); human vimentin (Zymed Laboratories, South San Francisco, CA); and human α-fetoprotein (Zymed Laboratories). Rabbit immunoglobulins against human von Willebrand factor VIII (Nordic Immunological Laboratories, Capistrano Beach, CA), human albumin (Protogen AG, Läufelfingen, Switzerland), and human fibrinogen (Zymed Laboratories) were also used. The cells were then incubated with biotinylated goat anti-mouse or anti-rabbit IgG (Life Technologies, Gaithersburg, MD), followed by fluorescein-conjugated streptoavidin (Amersham).

Extraction of Cells and Western Blot Analysis. Cells were harvested and lysed with 0.5 ml extraction buffer (1% Triton X100, 100 m*M* Tris-HCl, pH 8.0, 0.1 *M* NaCl). After centrifugation, aliquots of the supernatants were taken for determination of protein content by the method of Bradford (17), and the soluble proteins were used for Western blot analysis.

Western blot analysis was performed by electrophoretic transfer to nitrocellulose sheets (18) after SDS-PAGE (19). The sheets were incubated with mouse monoclonal antibodies to large T antigen and human α-fetoprotein as well as rabbit immunoglobulins against human albumin and human fibrinogen. Immunoreactive proteins were stained using an ABC kit (Vectastatin; Vector Laboratories, Burlingame, CA) as previously described (20).

Chromosomal Analysis. Karyotypic analysis by Giemsa banding (21) was carried out at the Life Information Analysis Center (LIAC; Komaki, Japan). Exact chromosome counts of 50 metaphases were made for ploidy determination.

Results

Establishment and Culture Characteristics of an NFL/T Cell Line. Hepatocytic cell lineage is preserved more than other cell types in our isolation and culture conditions (15). Cells were transfected with SV40(ori$^-$) DNA. One week after the transfection, only one expanding colony was observed. NFL/T cells have undergone up to 200 population doublings (PDs) without any culture crisis.

NFL/T cells grown as a monolayer on the collagen-coated dishes typically showed a cobblestone appearance at confluent stages (Fig. 1). Each cell included an ovoid nucleus with prominent nucleoli. Large Golgi area and many mitochondria, as well as lipid droplets, were seen in the cytoplasm (Fig. 2).

Growth Properties and Tumorigenicity. Figure 3 shows growth curves from cell populations at high and low densities. When cells were plated at high density, exponential cell growth was seen from Day 1 to Day 2. On the other hand, exponential cell growth was from Day 2 to Day 4 in the low-density population. Cell number reached plateau level on Day 4 regardless

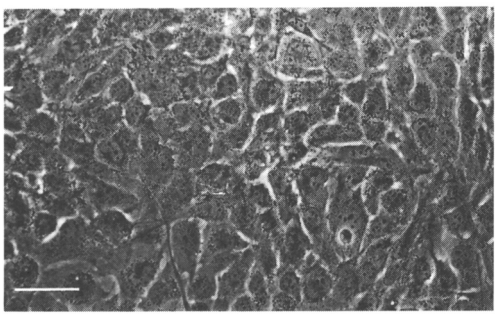

Figure 1. Phase-contrast microscopy of human fetal liver (NFL/T) cells. Bar: 10 μm.

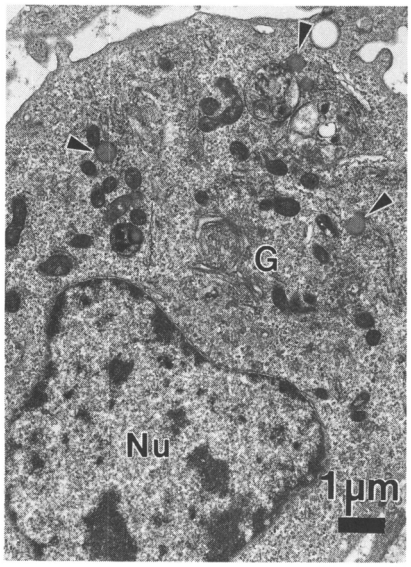

Figure 2. Electron microscopy of an NFL/T cell. Lipid droplets (arrowheads) are seen in the cytoplasm. N = nucleus, G = Golgi area. Bar: 1 μm.

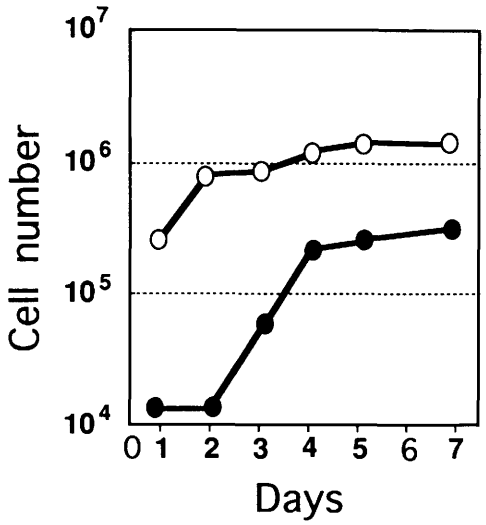

Figure 3. Density-dependent cell growth of NFL/T cells. Collagen type I-coated dishes (60 mm) were seeded with 4.0×10^5 cells (○) or 1.6×10^4 cells (●). Cells were counted using a hemocytometer.

of the initial density. We hypothesized this could be explained by cell-cell interaction.

Accordingly, cells were labeled with BrdU for 24 hr in various conditions. When cells were plated at high density, 100% of the cells incorporated BrdU from Day 1 to Day 2 (Fig. 4A). Low density cultures, allowed to reach this same density (4 days of culture), did not show 100% BrdU incorporation (Fig. 4B). Rather, BrdU was not incorporated by the cells in the center of the colony over a 24-hr period.

NFL/T cells (70 PDs) did not produce any tumors in six athymic nude mice over a period of 10 months.

Expression of SV40 T Ag. Indirect immunofluorescence showed anti-SV40TAg nuclear labeling in all

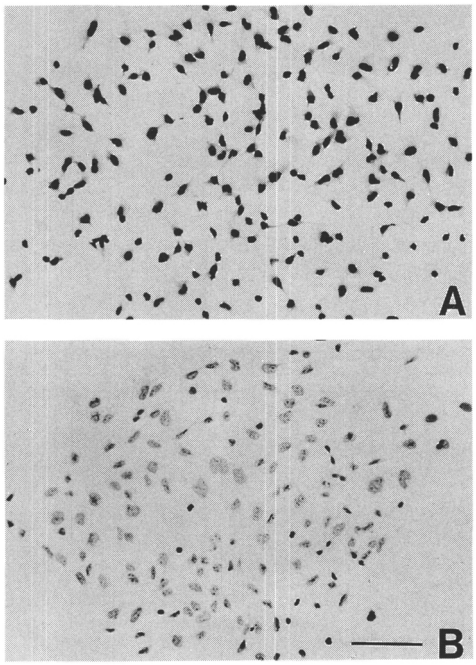

Figure 4. Light micrographs of proliferating cells. Cells were plated at 4.2×10^4 cells/cm^2 (A) or 1.7×10^3 cells/cm^2 (B). From Day 1 (A) or Day 4 (B), cells were incubated with BrdU for 24 hr. Incorporated BrdU was detected with peroxidase-labeled antibody technique. Cells were counter-stained with eosin. Bar: 20 μm.

NFL/T cells (Fig. 5A). Immunoblot analysis demonstrated the presence of SV40TAg protein with a molecular weight of 98 kd within the cell (Fig. 6, Lane 1).

Phenotype Characterization Using Antibodies. Albumin was detected in NFL/T cells by Western blot analysis (Fig. 6, Lane 2), while fibrinogen (Fig. 6, Lane 5) and α-fetoprotein (Fig. 6, Lane 3) were not detected. NFL/T cells showed positive immunofluorescence against monoclonal antibodies to cytokeratin 7 (CK 7) (Fig. 5B) and vimentin (Fig. 5C). Monoclonal antibody to CKs 8, 18, and 19 produced weak staining as intermediate filaments (data not shown). The cells did not immunoreact with polyclonal antibody to von Willebrand factor, a characteristic marker of endothelial cells (data not shown).

Chromosome Analysis. All the cells examined were hypo-tetraploid with apparent random trisomies (Fig. 7). Structural aberrations such as translocation and deletion (8–13) were not detected. The Y chromosome was not observed in any of 50 examined metaphases. These observations are consistent with autoptical findings.

Discussion

An immortalized cell line, NFL/T, was established after transfection of human fetal liver cells with a plasmid carrying SV40TAg. SV40TAg transfection is a promising technique for establishing a normal cell-like cell line and has been known to block the differentia-

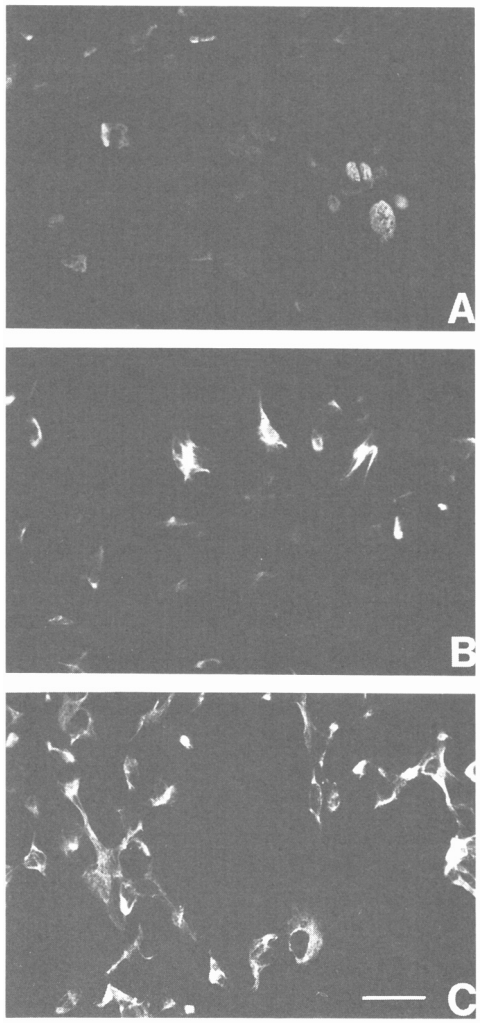

Figure 5. Immunofluorescent staining of SV40TAg (A), cytokeratin 7 (B), and vimentin (C). Bar: 10 μm.

tion process (22). However, altered growth control may be accompanied with an extended life span. The NFL/T showed normal cell-like growth more clearly than any other cell lines established in this method. The density-dependent proliferation of the NFL/T is comparable to the growth pattern of hepatocytes in primary cultures (23). Moreover, we studied "cell-cell" interaction (contact inhibition) of cell proliferation, shown by the loss of BrdU incorporation. These results may demonstrate the general growth characteristics of the liver, an organ which does not show continual self-renewal like skin and intestinal epithelia (4).

Morphologic features of NFL/T cells include some differentiated properties of hepatocytes, such as many mitochondria. Data from chromosomal analysis indicate NFL/T cells originated from a tetraploid cell. Since it is usually known that tetraploid cells are common in adult liver and the ploidy level increases after birth, our present data show that NFL/T contrast with the characteristic of fetal liver. However Pfeifer *et al.* showed that immortalized human adult cell line with transfection SV40TAg has diploid cells (24).

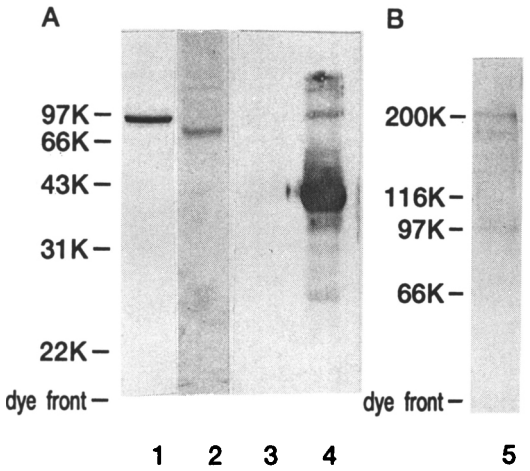

Figure 6. Immunoblot analysis after 10% (A) or 7.5% (B) SDS-PAGE. Each lane was loaded with 20 μl of the extract from 8 × 10^5 cells (Lane 1–3 and 5) or 100 ng of standard α-fetoprotein (Lane 4). Immunoreactive proteins were stained using an ABC kit with antibody to large T antigen (Lane 1), albumin (Lane 2), α-fetoprotein (Lane 3 and 4), or fibrinogen (Lane 5).

Positive immunoreactivity with albumin and cytokeratin 7 (CK 7) also suggests the differentiation of NFL/T cells. On the other hand, our data could not show the production of α-fetoprotein, a marker of fetal or abnormal liver, whose expression is known to precede albumin expression in the development of the fetal liver in rat (25). It is difficult to interpret the results of liver-specific protein expression from a clonal liver cell line. Van Eyken *et al.* (5) reported precise data of cytokeratin expression pattern in 56 human liver specimens from 6 weeks gestation to the 8th postnatal month: CK 7, which is expressed only in differentiated bile ducts, is negative until 20 weeks gestation, and only weakly positive until 1 month postnatally. Interestingly, NFL/T, which originated from a human fetus at the 18th week, displayed strong staining for CK 7. Our data also show strong staining for vimentin, which is typical for mesenchymal elements. However, previous reports (26–28) suggest it could be induced during the culture of liver epithelial cells. Our NFL/T cell line might be a pluripotential: it is important to clarify whether NFL/T cells are uncommitted cells, hepatocytes or bile duct epithelial cells (29). Enzyme activities clearly associated with hepatocytes might show cogent evidence for this cell line.

In summary, we have established an immortalized human fetal liver cell line by SV40TAg transfection. This cell line, NFL/T, showed normal cell-like growth properties and some differentiated phenotypes. Although further characterization is still needed such as the expression of α-fetoprotein, the NFL/T cell line might be promising for studying questions in developmental biology, oncology, hepatology, and perinatal medicine.

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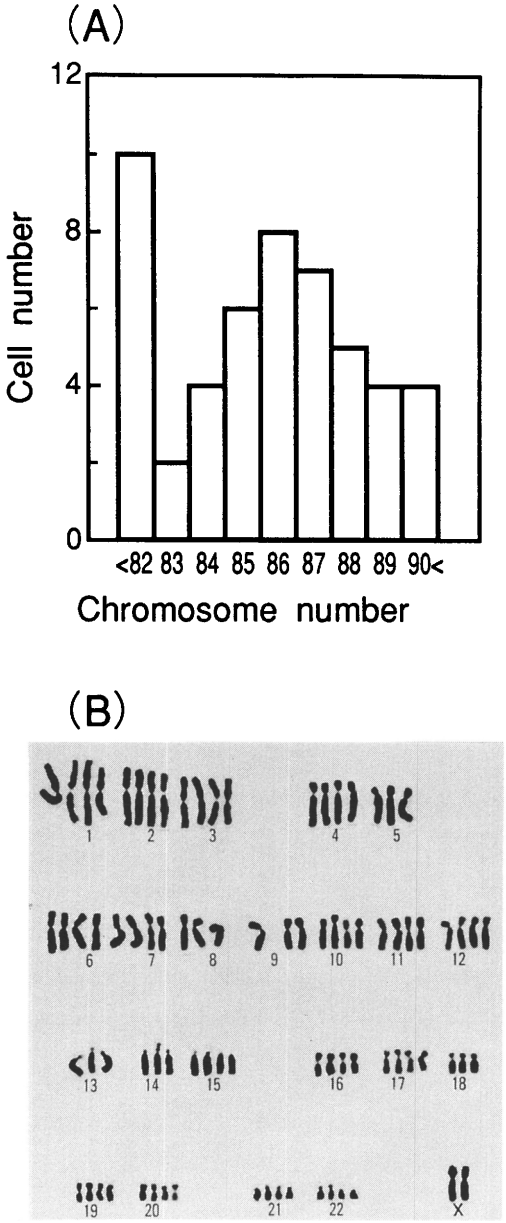

Figure 7. Chromosomal analysis of NFL/T cells (90 PDs). (A) Distribution of chromosome number. (B) G-banded karyotype with 84 chromosomes.

the SV40(ori$^-$) plasmid, and to Dr. Shinichi Magota and Mr. Hiroyasu Ohashi for the chromosomal analysis. Part of this work was supported by a Grant-in-Aid from the Ministry of Education of Japan.

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