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Induction of apoptosis in a human hepatocellular carcinoma cell line by a neutralizing antibody to transforming growth factor-α

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Abstract A cell line derived from a Japanese man with hepatocellular carcinoma was established in culture and designated OCUH-16. The cell line has the morphological and chromosomal features of hepatocellular carcinoma cells and has a short doubling time (~33 h). OCUH-16 cells were shown to express transforming growth factor-α (TGF-α) in addition to albumin, DNA polymerase-α, c-JUN, and the retinoblastoma gene product. Electron microscopy revealed TGF-α immunoreactivity associated with the cell membrane, but TGF-α was not detected in medium conditioned by OCUH-16 cells by enzyme-linked immunosorbent assay. Reverse transcription and polymerase chain reaction analysis revealed the presence of TGF-α messenger RNA in these cells. Culture of OCUH-16 cells in the presence of a neutralizing antibody to TGF-α inhibited cell proliferation and induced many cells to undergo apoptosis (programmed cell death). These observations suggest that endogenous TGF-α is necessary for OCUH-16 cell growth.

Key words Hepatocellular carcinoma · Apoptosis · Transforming growth factor-α

Introduction

Hepatocellular carcinoma (HCC) is a common neoplasm whose prevalence is particularly high in Asia, including Japan, and sub-Saharan Africa [26, 37, 44]. The aetiological agents for this condition appear to differ in different countries. The isolation of hepatitis C virus (HCV) in 1989 led to the development of a recombinant antigen-based immunoassay for the detection of antibodies to HCV (HCV-Ab) [9, 24]. HCV-Ab circulating in the blood can thus be assayed, and a relationship between chronic HCV infection and the occurrence of HCC associated with cirrhosis has been demonstrated in various countries [6, 12, 13, 18, 22, 34, 46, 47]. We previously established an original HCC cell line (designated OCUH-16) [41], derived from a patient with HCV-Ab, for use in studies of potential treatments and oncogenesis. We now describe an important role for transforming growth factor-α (TGF-α) in the proliferation of OCUH-16 cells.

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Materials and methods

We established the HCC cell line from a Japanese patient with HCV-Ab (second-generation assay for HCV-Ab; HCV genotype type III according to the classification of Okamoto et al. [36]). The HCC was in the right lobe of the liver and measured 70 by 76 mm. The biopsy specimen was classified as grade I according to the classification of Edmondson and Steiner [16]. With the use of immunocytochemistry with antibodies to human albumin, c-JUN [1, 5], DNA polymerase-α [4, 30], and the retinoblastoma gene product [25, 33], we showed that some OCUH-16 cells expressed these proteins. Electron microscopy of OCUH-16 cells revealed the presence of junctional complexes, microvilli, and many organelles. Chromosomal analysis by G-banding method showed that the number of chromosomes in OCUH-16 cells varied in number from 61 to 83, many of which were abnormal. The doubling time of these cells was about 33 h. The tumour markers α-fetoprotein, carcinoembryonic antigen, and the protein induced by vitamin K antagonist II were not detected. Transplantation of OCUH-16 cells in triplicate into nude mice did not result in the formation of solid tumours.

OCUH-16 cells were cultured in William's Eagle medium containing 10% fetal bovine serum, 0.1 mmol/l insulin, and 10 mmol/l dexamethasone in 250-ml flasks (Sumilon; Sumitomo, Akita, Japan) under 5% CO_2 in air.

In order to demonstrate TGF-α 1×10^4 cells were transferred to a four-well Lab-Tek chamber slides. After 5 days, the cells were fixed with periodate, lysine, and 2% paraformaldehyde, rinsed with 10 mmol/l phosphate-buffered saline (PBS), and incubated

overnight in 0.1 mmol/l sodium phosphate buffer containing 20% (w/v) sucrose. After rinsing with PBS, the cells that adhered to the slide were incubated overnight at 4°C with a mouse monoclonal antibody to TGF-α (Ab2; Oncogene Science, NY, diluted 1:1000 in PBS) or with normal mouse immunoglobulin G (IgG) (control) (Serotic, UK). Endogenous peroxidase activity was inhibited by incubating with methyl alcohol containing 0.03% hydrogen peroxide, after which they were rinsed with PBS and incubated for 6 h at 4°C with horseradish peroxidase-conjugated goat polyclonal antibodies to mouse IgG. The cells were finally treated with a solution containing 0.25% 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.03% hydrogen peroxide, and counterstained for nuclei with 10% methyl green. TGF-α immunoreactivity was detected as brown staining by light microscopy.

For ultrastructural localization of TGF-α, 1×10^4 OCUH-16 cells were transferred to a four-well chamber slide and processed as described for light microscopy, with the exception that, after incubation with the peroxidase-conjugated goat polyclonal antibodies to mouse IgG, the cells were postfixed with 0.5% glutaraldehyde for 5 min and then treated with 0.25% DAB in PBS for 30 min followed by 0.25% DAB solution containing 0.03% hydrogen peroxide for 10 min. The cells were finally fixed again with 1% osmium tetroxide for 30 min at 4°C, dehydrated with a graded series of ethanol solutions, embedded in Epon 812, and examined by electron microscopy.

To demonstrate TGF-α by immunoblot analysis OCUH-16 cells were suspended in a lysis buffer [1% Triton X-100, 2 mmol/l sodium vanadate, 50 mmol/l tris-HCl (pH 7.6), 2 mmol/l phenylmethylsulfonyl fluoride, 40 μg/ml leupeptin, 40 μg/ml aprotinin, 40 μg/ml soybean trypsin inhibitor after 7 days in culture and centrifuged at 400 *g* for 5 min. The supernatant was centrifuged again at 100,000 *g* for 30 min, and the new supernatant was collected, mixed with sodium dodecyl sulfate (SDS) sample buffer [1% SDS, 5% (w/v) sucrose, 2.5% (v/v) glycerol, 60 mmol/l Tris-HCl (pH 6.8), 0.01% bromophenol blue, 0.05 mol/l dithiothreitol], and subjected to electrophoresis on a 15%–25% polyacrylamide gradient gel. The separated proteins were transferred to a polyvinylidine difluoride membrane, which was then stained with mouse monoclonal antibody Ab2 or normal mouse IgG (control) and the avidin–biotin–peroxidase complex (ABC) method.

RNA isolation and reverse transcription and polymerase chain reaction (RT-PCR) assay for TGF-α mRNA was carried out as follows. Total RNA was extracted from OCUH-16 cells after 4 days in culture by the acid guanidinium thiocyanate-phenol-chloroform method [8]. Poly $(A)^+$ RNA was purified with a mRNA purification kit (Perkin-Elmer, Norwalk, Conn.) and cDNA was synthesized from 2 μg of mRNA with SuperScript reverse transcriptase (Gibco BRL, Gaithersburg, Md.) and oligonucleotide primers. PCR was performed with the sense primer 5′-ATGGTCCCCTCGGCTGGACAG-3′ (nucleotides 35 to 55, 21 mer), and the antisense primer 5′-GGCCAGCTTCTTCTGGCTGGCA-3′ (nucleotides 310 to 331, 22 mer) for 35 cycles of 94°C for 1 min, 50°C for 2 min, and 72°C for 2 min. The PCR products obtained were separated by elecrophoresis and stained with ethidium bromide.

OCUH-16 cells (10×10^4 per well) were cultured in William's Eagle medium containing 10% fetal bovine serum in 250-ml culture flasks. The culture medium was collected after 2, 5, and 7 days and assayed for TGF-α with an enzyme-linked immunosorbent assay quantitative kit (Oncogene Science).

After 5 days in culture in four-well Lab-Tek chamber slides, OCUH-16 cells were processed for immunostaining with a mouse monoclonal antibody to the EGF receptor (Upstate Biotechnology, Lake Placid, N.Y.), diluted 1:100 in PBS or normal mouse IgG (control) as described for TGF-α immunostaining.

In a further part of the study OCUH-16 cells (1×10^4 per well) were transferred to four-well chamber slides with William's Eagle medium. A neutralizing monoclonal antibody to TGF-α (5 μg/ml) (Ab3 without NaN_3; Oncogene Science) or normal mouse IgG was added to the cells, which were subsequently fixed with periodate, lysine, and 2% paraformaldehyde and stained with haematoxylin and eosin (H&E) after 1, 2, 3, 5, and 7 days, and the adherent cells were counted. For each experimental condition, cells in 10 fields at a magnification of ×100 were counted and the results expressed as means ± standard deviation (SD). In a separate experiment, cells (1×10^4 per well) were cultured in four-well chamber slides with William's Eagle medium for 3 days before addition of the mouse monoclonal antibody Ab3 (5, 2.5, or 0.1 μg/ml) or normal mouse IgG. After an additional 24 h in culture, the cells were fixed, stained with H&E, and counted.

We also examined OCUH-16 cells that had been treated with antibody Ab3 by transmission electron microscopy. After 3 days of culture, the cells were incubated with the antibody (5 μg/ml) for 24 h and then rinsed with 10 mmol/l sodium phosphate buffer containing 20% sucrose for 6 h. The cells were then fixed with 1.5% glutaraldehyde for 20 min and with 2% osmium tetroxide for 1 h, dehydrated with a graded series of ethanol solutions, and embedded in Epon 812. Sections 60–90 nm thick were stained with uranyl acetate and lead citrate.

Finally, we examined the induction of apoptosis in OCUH-16 cells by antibody Ab3 biochemically. After 3 days of culture in 250-ml culture flasks, cells were incubated with antibody (5 μg/ml) or normal mouse IgG for 24 or 48 h. The cells were then suspended in an ice-cold solution containing 10 mmol/l Tris-HCl (pH 8.0), 20 mmol/l ethylenediaminetetraacetic acid (EDTA), and 0.25% Triton X-100 and mixed by inversion. After incubation on ice, the cell lysate was centrifuged at 5000 *g* for 10 min. The supernatant was collected and DNase-free RNase (Sigma, St. Louis, Mo.) was added to give a final concentration of 20 μg/ml. After incubation for 30 min at 37°C and addition of an equal volume of 1% SDS, the mixture was further incubated for 10 min at 55°C. Small DNA molecules were extracted with a 1:1 mixture of phenol and chloroform, precipitated by ethanol in the presence of 0.1 volume of 3 mol/l sodium acetate (pH 5.2), and subjected to electrophoresis in a 1.7% agarose gel in 40 mmol/l Tris-acetate (pH 8.0) containing 1 mmol/l EDTA. The gel was stained with ethidium bromide and photographed under ultraviolet light.

Results

TGF-α immunoreactivity was apparent as brown granules in the cytoplasm mostly in the perinuclear region of OCUH-16 cells (Fig. 1A). After 3 days of cell culture, 30 (15%) of the 202 cells examined were stained; after 5 days, 70 (20%) of 350 cells were stained; and after 7 days, 36 (15%) of 246 cells were stained. Electron microscopy revealed that TGF-α immunoreactivity was localized to cell membranes (Fig. 1B). Immunoblot analysis with monoclonal antibody Ab2 of OCUH-16 cells cultured 7 days revealed three immunoreactive bands of 70, 35, and 20 kDa (data not shown).

RT-PCR analysis revealed the expected 297-bp product of TGF-α mRNA from OCUH-16 cells after 4 days in culture (Fig. 1C). TGF-α was not detected in the conditioned medium of OCUH-16 cells by enzyme-linked immunosorbent assay after 2, 5, or 7 days of culture. Immunostaining for the EGF receptor revealed the presence of immunoreactivity associated with the cell membrane in many cells (Fig. 1D).

After 3 days of culture and exposure to the Ab3 neutralizing antibody to TGF-α for 24 h, many OCUH-16 cells appeared shrunken, with dark cytoplasm and condensed basophilic nuclei (Fig. 2A), all of which are characteristics of apoptosis [19, 23, 52]. Some cells had detached from the slide and were floating in the medium, whereas others were not shrunken but were large and

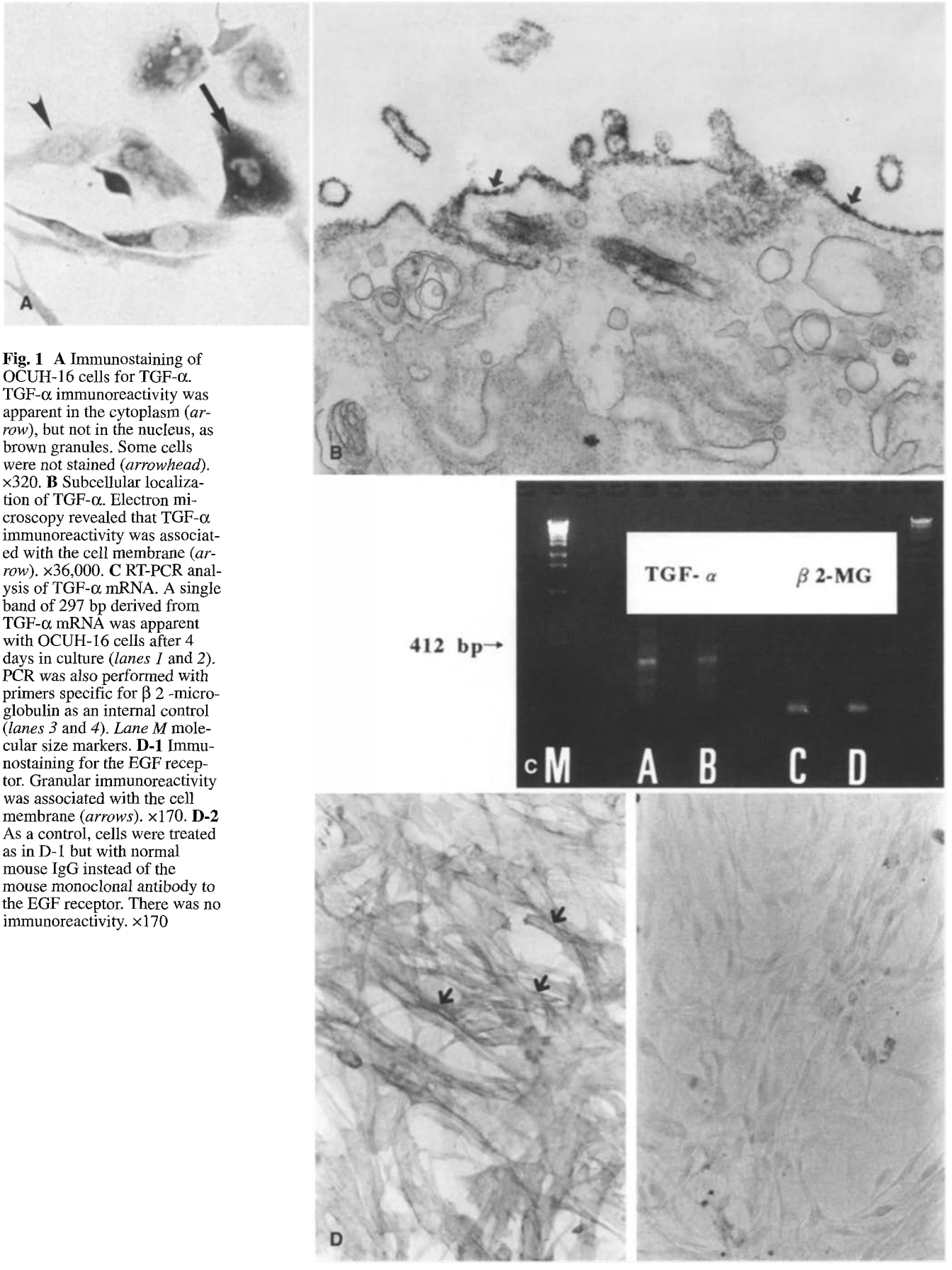

Fig. 1 **A** Immunostaining of OCUH-16 cells for TGF-α. TGF-α immunoreactivity was apparent in the cytoplasm (*arrow*), but not in the nucleus, as brown granules. Some cells were not stained (*arrowhead*). ×320. **B** Subcellular localization of TGF-α. Electron microscopy revealed that TGF-α immunoreactivity was associated with the cell membrane (*arrow*). ×36,000. **C** RT-PCR analysis of TGF-α mRNA. A single band of 297 bp derived from TGF-α mRNA was apparent with OCUH-16 cells after 4 days in culture (*lanes 1* and *2*). PCR was also performed with primers specific for β 2 -microglobulin as an internal control (*lanes 3* and *4*). *Lane M* molecular size markers. **D-1** Immunostaining for the EGF receptor. Granular immunoreactivity was associated with the cell membrane (*arrows*). ×170. **D-2** As a control, cells were treated as in D-1 but with normal mouse IgG instead of the mouse monoclonal antibody to the EGF receptor. There was no immunoreactivity. ×170

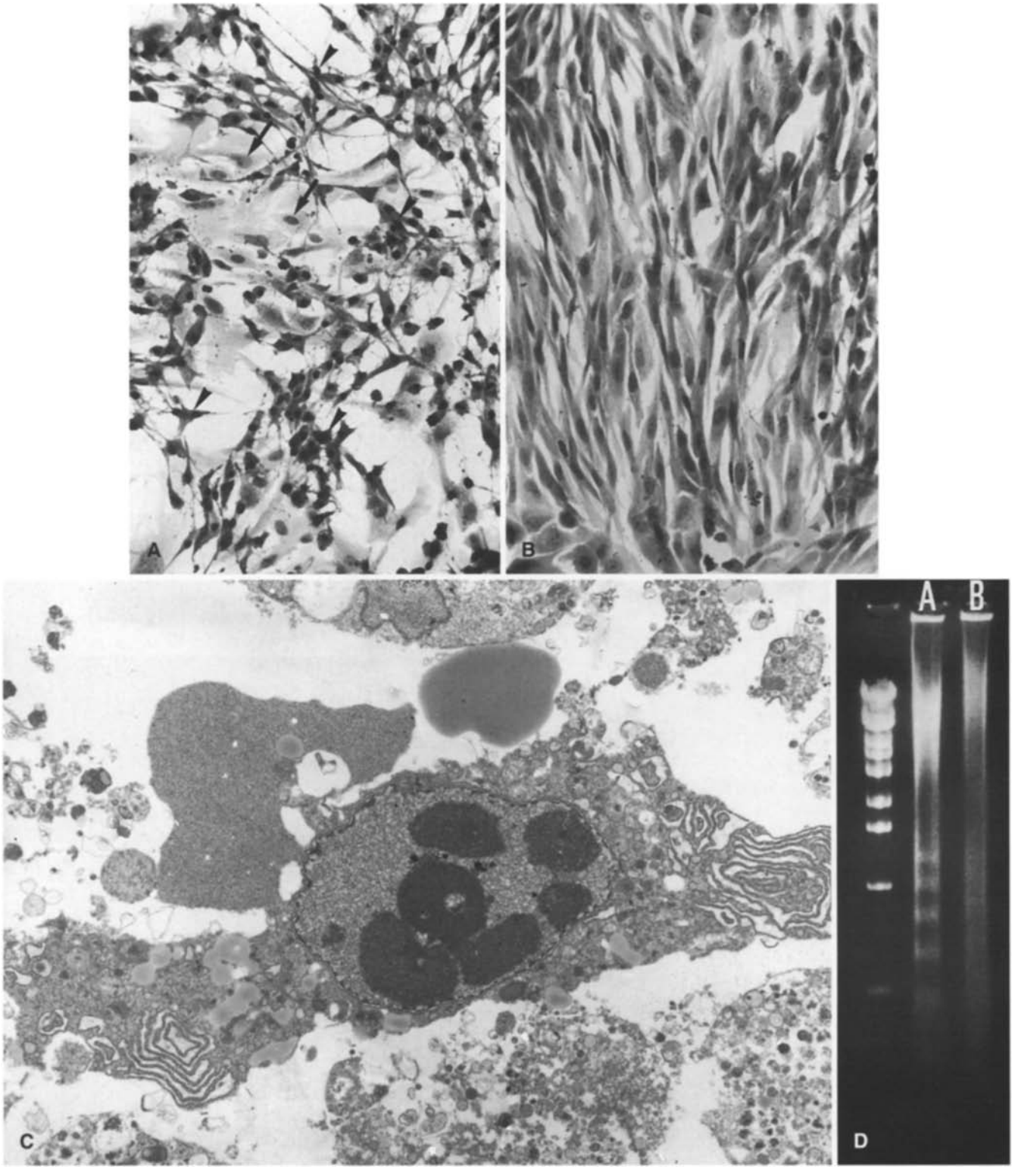

Fig. 2 **A** Effect of a neutralizing antibody to TGF-α on OCUH-16 cell morphology. Cells were cultured for 3 days and then exposed to antibody Ab3 (5 μg/ml) for 24 h. Many cells are shrunken, with dark cytoplasm and condensed nuclei (*arrowheads*). A few large, spindle-shaped cells with nuclei that are not condensed are also apparent (*arrows*). H & E, ×170. **B** As a control, cells were treated as in **A** but with normal mouse IgG instead of the neutralizing antibody. Most cells contain nuclei that are not condensed and abundant cytoplasm. H & E, ×170 **C** Electron micrograph of a shrunken OCUH-16 cell after treatment with the neutralizing antibody. Most of the chromatin has aggregated into granular masses. Loss of some microvilli and dilatation of the endoplasmic reticulum are also apparent. A protuberance can be seen on the cell surface. ×6,800. **D** Gel electro-phoresis of DNA from OCUH-16 cells that had been cultured for 3 days and then exposed to the neutralizing antibody (5 μg/ml) for 24 h (*lane A*) or 48 h (*lane B*). *Lane* in left molecular size markers

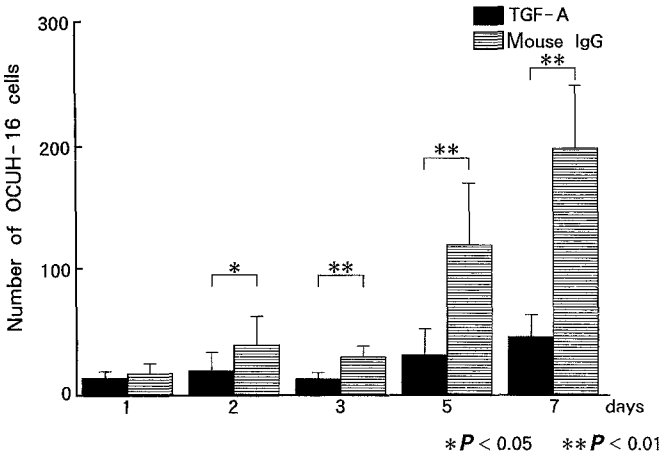

Fig. 3 Number of surviving OCUH-16 cells after culture for the indicated times with the neutralizing antibody to TGF-α or normal mouse IgG. Data are mean ± SD for ten fields (each field, 0.08 mm^2). *$P < 0.05$, **$P < 0.01$ (Student's t-test). ×100

spindle shaped, with basophilic cytoplasm and nuclei that were not condensed. These latter cells frequently formed clusters with each other in areas containing shrunken cells. Most of the cells treated with normal mouse IgG were not shrunken, and contained large nuclei and abundant cytoplasm (Fig. 2B); few showed apoptotic features. Electron microscopy of the shrunken cells treated with Ab3 revealed that both cytoplasm and nuclei had shrunk and most chromatin had aggregated into compact granular masses (Fig. 2C). Other changes included the loss of some microvilli, the formation of blunt protuberances on the cell surface, the appearance of many translucent cytoplasmic vacuoles, and dilatation of the cisternae of the endoplasmic reticulum. Again, these changes are characteristic of apoptosis [19, 23, 52].

Gel electrophoresis of DNA prepared from nuclei of OCUH-16 cells that had been cultured for 3 days and exposed to Ab3 (5 μg/ml) for 24 h revealed a ladder pattern of fragments that consisted of multimers of 180 bp; treatment of cells with the antibody for 48 h resulted in a smeared pattern of fragments (Fig. 2D). Treatment of cells with normal mouse IgG did not induce DNA fragmentation (data not shown).

When cells were cultured with the neutralizing antibody to TGF-α (5 μg/ml) or normal mouse IgG for various times, the number of unshrunken cells was significantly greater after 2, 3, 5, or 7 days with normal IgG than Ab3 (Fig. 3). When cells were cultured for 3 days and then treated for 24 h with normal mouse IgG or with Ab3 at concentrations of 0.1, 2.5, and 5 μg/ml, the mean percentage of apoptotic cells was 6.5 ± 1.6, 7.2 ± 1.2, 25.7 ± 5.3, and 80.1 ± 2.5, respectively.

Discussion

The OCUH-16 cell line exhibits many characteristics of HCC cell [41] confirming that it was, derived from a human HCC. Of the many growth factors active on hepatocytes, EGF, TGF-α, and TGF-β are prominently associated with cell proliferation, hepatic regeneration, and hepatic tumorigenesis via autocrine, paracrine, and juxtacrine mechanisms [2, 14, 28, 31, 43]. Both TGF-α and TGF-β are produced in the liver, but EGF is not. TGF-α is expressed by hepatocytes during liver regeneration and in human HCC cells, and it is a potent mitogen for rat hepatocyte cell lines, whereas TGF-β may either stimulate or inhibit the growth of such cells, depending on its concentration and the specific cell type [7, 20, 29, 39, 40, 49, 50]. In transgenic mice that overproduce TGF-α, this growth factor was abundant in precancerous hepatocellular foci, adenomas and HCC than in the hepatocytes of the surrounding parenchyma [21, 45]. Therefore, we chose to examine the effects of TGF-α on OCUH-16 cells. We demonstrated the presence of TGF-α mRNA and protein in OCUH-16 cells, although the growth factor did not appear to be revealed into the culture medium in detectable amount. Electron microscopy revealed TGF-α immunoreactivity associated with the cell membrane, suggesting that OCUH-16 cells express the membrane-bound precursor form of TGF-α, which stimulate OCUH-16 cell proliferation via juxtacrine mechanism. Moreover, Barrandon et al. reported that TGF-α might be looked as migration factor in keratinocytes [3]. A similar phenomenon may occur in HCC cells. The precursor of TGF-α, pro-TGF-α, promotes cell proliferation via the EGF receptor [2, 14, 21, 31]. Immunoblot analysis of OCUH-16 cells with antibodies to TGF-α revealed three immunoreactive bands; the 20-kDa band may correspond to pro-TGF-α, whereas the 35- and 70-kDa bands may correspond to polymerized or glycosylated pro-TGF-α [14, 50]. Inactivation of growth factors by neutralizing antibodies induces apoptosis in many cell lines, including those derived from haematopoietic cells, neurons, breast cancer, and prostate cancer [11, 15, 32, 38, 42]. Lin and Chou [27] and Fukuda et al. [17] showed that TGF-β induced apoptosis in human and rat HCC cell lines, whereas Oberhammer et al. [35] showed that this growth factor induced apoptosis in rat hepatocytes and regenerating liver. With the use of light and electron microscopy as well as DNA electrophoresis [10, 51], we have now shown a neutralizing antibody to TGF-α induces of apoptosis of OCUH-16 cells in dose-dependent manner, indicating the importance of endogenous TGF-α for the survival of these cells. The induction of apoptosis in HCC cells by inactivation of TGF-α may be applicable to clinical treatment, given that Hsia et al. [20] have shown that TGF-α is expressed by 80% of HCC.

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