

Transforming Growth Factor- α Induces the Differentiation of Sarcomatoid Cholangiocarcinoma Cells

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A sarcomatoid cholangiocarcinoma cell line, ETK-1, was established from a patient. Phenotypically, the cells corresponded to immature biliary epithelial cells. Because a small number of ETK-1 cells appeared to differentiate spontaneously along a biliary epithelial lineage in continuous culture, we examined the factors that initiate and/or promote the differentiation of the cells. Transforming growth factor- α (TGF α) induced significant changes in ETK-1 cells. After stimulation with the factor, ETK-1 cells displayed morphologic transformation at a much higher frequency, with the appearance of many large cells with intracytoplasmic vacuoles, and the production of mucinous substances. These morphologically transformed cells were phenotypically similar to well-differentiated adenocarcinoma cells. The expression pattern of integrins after TGF α treatment also supported the maturation of the ETK-1 cells. The antibody against the receptor of TGF α inhibited these changes by TGF α . Moreover, the proliferation rate of ETK-1 cells was suppressed by TGF α . Our data suggest that TGF α can act as a differentiation factor along a biliary epithelial lineage.

Key words: Cholangiocarcinoma — Biliary epithelial cell — Differentiation — TGF α — Liver stem cell

Many investigators support the existence of bipotential liver stem cells which can differentiate into both hepatocytes and biliary epithelial cells (BECs).^{1–3} “Oval cells,” which have been demonstrated in the adult liver in some model animals,^{3–7} are regarded as a population of liver stem cells. However, the mechanism initiating or promoting the differentiation of the stem cells is not known. Many factors in the intrahepatic microenvironment, e.g., cytokines, cell adhesion molecules, and the extracellular matrix, may affect the differentiation. Transforming growth factor- α (TGF α), which is a polypeptide produced by many cell types including transformed cells, is one of the cytokines that act on both hepatocytes and nonparenchymal liver cells. TGF α is structurally related to epidermal growth factor (EGF), and binds to the same receptor as EGF.⁸ Although TGF α is a principal regulator of normal growth in epithelial tissues, many other functions of TGF α have been identified, such as early fetal development,^{9–11} regeneration and transformation,^{12–14} invasion and homing,¹⁵ and neovascularization.¹⁶ However, no direct evidence that TGF α can induce differentiation or metaplastic changes of bile duct cells has been reported.

We have established and reported a human sarcomatoid cholangiocarcinoma (CC) cell line with bipotential differentiation activity. ETK-1 cell line is derived from rhabdoid-type sarcomatoid CC cells.¹⁷ After 5-azacytidine

treatment, ETK-1 cells converted into hepatocytes, and by subcutaneous inoculation of ETK-1 cells, ductular well-differentiated CC appeared. We have also established another human sarcomatoid CC cell line, SSP-25, which originated from spindle-type sarcomatoid CC cells.¹⁸ Sarcomatous change in the liver is considered as a result of dedifferentiation.^{19,20} In fact, we found that a small number of these sarcomatoid CC cells showed spontaneous morphologic and phenotypic transformation into a maturer stage in continuous culture. In order to clarify the mechanism of these changes, we examined the response of the sarcomatoid cells to some possible cytokines. We report here that TGF α can act as a differentiation-inducer along a biliary epithelial lineage.

MATERIALS AND METHODS

Cell lines and culture medium A human sarcomatoid CC cell line, ETK-1, was derived from rhabdoid-shaped sarcomatoid cells.^{17,21} MEK cell line was derived from converted cells which emerged after 5-azacytidine treatment to ETK-1 cells.¹⁷ The hepatocellular carcinoma (HCC) cell line HepG2, the CC cell line H-1 (provided by the First Department of Surgery, Okayama University School of Medicine, Okayama),²² the fibroblast cell line WI-38, and the melanoma cell line MMJ-2 (established in our laboratory) were also used as controls. The cell lines were cultured in MA medium (RPMI-1640, Sigma, St. Louis, MO) containing 10 mM HEPES buffer (Life Technologies, Grand Island, NY), 2 mM L-glutamine (Flow

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Labs, McLean, VA), 0.1 mM nonessential amino acids (Life Technologies), 1 mM sodium pyruvate (ICN Biochemicals, Costa Mesa, CA), 5×10^{-5} M β -mercaptoethanol (Sigma), and 10% (v/v) fetal bovine serum (Life Technologies).

TGF α and acidic fibroblast growth factor (aFGF) treatments ETK-1 and MEK cells were plated on 100 mm culture dishes (10^5 cells/dish) and treated with various concentrations of TGF α (Oncogene Science, Manhasset, NY) or aFGF (Oncogene Science) in MA medium. Morphologic changes were checked every day. The medium was replaced every 2 days. For the competitive assays, these cells were pretreated with 1 μ g/ml of anti-EGF-receptor monoclonal antibody (MoAb) (Seikagaku Corp., Tokyo) 6 h before the addition of TGF α . The mitogenic effect of these factors was examined using a Cell Proliferation Assay Kit (Promega, Madison, WI), as described by the manufacturer.

Immunocytochemistry Immunocytochemical staining was performed with the avidin-biotin peroxidase complex method, as described by the manufacturer (Histofine SAB-PO Kit, Nichirei, Tokyo). The following MoAbs and antisera were used: anti-vimentin MoAb (1:100 dilution, Novocastra, Newcastle, UK), anti-carcinoembryonic antigen (CEA) MoAb (1:100 dilution, Novocastra), anti- α -fetoprotein (AFP) antiserum (1:1000 dilution, Dako, Copenhagen, Denmark), anti-albumin antiserum (1:1000 dilution, Dako), anti- γ -glutamyl transpeptidase (GGT) MoAb (1:100 dilution, Cosmo Bio, Tokyo), anti-cytokeratin (CK) 18 MoAb (1:100 dilution, Novocastra), anti-CK19 MoAb (1:100 dilution, Novocastra), anti-carbohydrate antigen 19-9 (CA19-9) MoAb (1:200 dilution, Novocastra), and anti-c-Met antiserum (1:100 dilution, Oncogene Science). The periodic acid-Schiff (PAS) reaction was carried out using a PAS Staining Kit as described by the manufacturer (Muto Chemical, Tokyo).

Flow cytometric analysis The cultured cells were detached from the dishes using 0.25% trypsin and 1 mM EDTA, and were washed with phosphate-buffered saline (PBS) and resuspended in PBS containing 0.05% NaN₃ and 2% fetal bovine serum. The cells were incubated with the following MoAbs (1:200 dilution) at 4°C for 30 min: anti-EGF receptor MoAb, anti-integrin α_2 , α_3 , and α_6 MoAbs (Immunotech, Marseille, France), and anti-c-Kit MoAb (Oncogene Science). The cells were then washed three times with PBS containing 0.1% NaN₃, incubated with a fluorescein-conjugated goat anti-mouse IgG antibody (Cappel, Durham, NC) at 4°C for 30 min, washed three times, and then analyzed with an EPICS ELITE flow cytometer (Coulter Electronics, Hialeah, FL) using standard techniques. As a negative control, preimmune rabbit serum was used.

RNA extraction and northern blot analysis Total RNA was isolated from cultured cell lines by means of the

guanidine thiocyanate method,²³⁾ and analyzed by northern blotting. RNA samples (10 μ g each) were electrophoresed on 0.75% agarose-6% formaldehyde gels and then transferred onto nylon membranes. Probes for TGF α (Oncogene Science) were commercially obtained. A β -actin probe was used as a control. The β -actin probe was labeled with [α -³²P]dCTP using oligo-primed labeling, and the TGF α probe was labeled with [γ -³²P]ATP using the end-labeling method.

Reverse transcriptase-polymerase chain reaction (RT-PCR) For each reaction, 5 μ g of total RNA was reverse-transcribed to complementary DNA (cDNA) using a cDNA Synthesis Kit (Takara, Kyoto). The resulting cDNA was then subjected to 40 cycles of PCR using the Program Temperature Control System (Astec, Fukuoka), *Taq* DNA polymerase (Promega), and primers for human hepatocyte growth factor (HGF), aFGF, FGF-receptor, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The set of primers for HGF, which give an amplified fragment of 387 base pairs, consisted of a forward primer of 5'CTATGCAGAGGGACAAAGGAAAAGA3' and a reverse primer of 5'GGCAAAAAGCTGTGTTTCGTGTGGTA3'. The set of aFGF primers consisted of a forward primer of 5'CTAACAGGTGGGTGGGTATG3' and a reverse primer of 5'CTCTGGGGTGCTTTCTTTCT3', giving a fragment length of 226 base pairs. The FGF-receptor primers consisted of a forward primer of 5'AAGGACAAACCCAAACCGTGTGACC3' and a reverse primer of 5'GCCAAAGTCTGCTATCTTCATCAC3', giving a fragment of 425 base pairs. The GAPDH primers consisted of a forward primer of 5'GATTTGGTTCGTATTGGGCGC3' and a reverse primer of 5'ACGTACTCAGCGCCAGCATC3', giving a fragment of 416 base pairs. After amplification, each sample was applied to a 1% agarose/ethidium bromide gel at 100 V. The resulting gel was photographed with ultraviolet illumination.

RESULTS

Spontaneous transformation of ETK-1 cells ETK-1 and MEK cell lines were used in this study (see "Materials and Methods"). The HCC cell line HepG2 and CC cell line H-1 were also used as controls. Phenotypic characterization of these cell lines is presented in Table I. When ETK-1 cells proliferate to high cell density, a small number of larger mucin-producing cells with intracytoplasmic vacuoles can be identified (Fig. 1 and Table II). CEA and CA19-9 are identified in these larger cells by immunocytochemistry, but vimentin is not (data not shown). However, these cells could not be isolated and purified because of their small number and low proliferation rate. After single cell recloning of ETK-1 by the limiting dilution method, every clone obtained exhibited the same morphologic and phenotypic transformation in continuous culture. This

eliminated the possibility that the original ETK-1 cells contained more than one cell type and represented a mixed culture. This phenomenon demonstrates the ability of ETK-1 cells to convert spontaneously into mucin- and CEA-producing cells which may be in a more differentiated stage. On the other hand, MEK cells are morphologically and phenotypically homologous.

Table I. Immunocytochemical Analysis of Morphologically Transformed ETK-1 Cells after TGF α Treatment and Comparison with Original ETK-1, MEK, HepG2, and H-1 Cells

	Transformed ETK-1 (+TGF α)	ETK-1	MEK	HepG2	H-1
GGT	+	+	+	+	+
CK18	+	+	+	+	+
CK19	+	+	+	+	+
AFP	-	-	+	+	-
Albumin	-	-	+	+	-
Fas antigen	-	-	+	+	-
TPO	-	-	+	+	-
Integrin α 1	-	-	+	+	-
CEA	+	-	-	-	+
CA19-9	+	-	-	-	+
Vimentin	-	+	-	-	-
Mucin	+	-	-	-	+

Percentage of positive cells: +>80% >+/->10% >-.

TGF α , transforming growth factor- α ; GGT, γ -glutamyl transpeptidase; CK, cytokeratin; AFP, α -fetoprotein; TPO, thrombopoietin; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9.

Growth factor/receptor systems in ETK-1 and MEK cells Because a few ETK-1 cells showed spontaneous morphologic and phenotypic changes, we suspected that some factor(s) may induce differentiation along a biliary epithelial lineage through an autocrine mechanism. Thus, we examined the expression of some kinds of cytokines, HGF, TGF α , stem cell factor (SCF), and aFGF, and their receptors in ETK-1 and MEK. HepG2 and the well-differentiated CC cell line H-1 were also examined as controls. The expression of HGF receptor (c-Met) was tested by immunocytochemistry (data not shown). The expression of

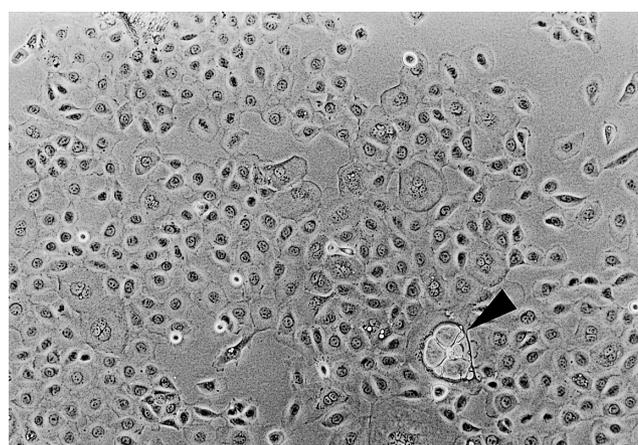


Fig. 1. Phase contrast microscopy (original magnification, $\times 50$). At high cell density, a few mucin-producing cells emerge among the original ETK-1 cells (arrow).

Table II. Morphologic and Phenotypic Changes in ETK-1 Cells

Media additions	Morphologically converted cells ^{a)} (per 1000 cells)	Positive cells ^{b)} (per 1000 cells)		
		PAS	CEA	CA19-9
No stimulation	0.2 \pm 0.1	0.3 \pm 0.1	0.5 \pm 0.2	0.3 \pm 0.2
TGF α	66.0 \pm 15.8	94.6 \pm 5.9	294.4 \pm 35.6	260.0 \pm 23.3
Anti-EGFR+TGF α	2.2 \pm 0.6	13.0 \pm 1.3	43.5 \pm 10.4	30.2 \pm 5.6
aFGF	0.4 \pm 0.1	1.8 \pm 0.3	2.1 \pm 0.4	0.7 \pm 0.6

TGF α , transforming growth factor- α ; aFGF, acidic fibroblast growth factor; EGFR, epidermal growth factor receptor.

Values reported are average \pm SD.

a) Larger-size cells with intracytoplasmic vacuoles. Method: 10^5 ETK-1 cells were plated on 60 mm culture dishes with a grid. At 24 h after the plating, TGF α or aFGF was added (final concentration; 10 ng/ml). For competition assay, anti-EGFR MoAb pretreatment (final concentration; 1 μ g/ml) was done 6 h before TGF α -stimulation. At 48 h after stimulation, the cell number was counted on photographs of six fields in two independent dishes. Each field contained over 1500 cells. Cells were finally in a subconfluent state.

b) 10^3 ETK-1 cells were seeded on chamber slides (Nunc, Naperville, IL). Twenty-four hours after the plating, cytokines and antibody were added as above. At 48 h after stimulation, the cell number in chamber was counted directly under a microscope. Four chambers were prepared for each case. Cells were finally in a subconfluent state.

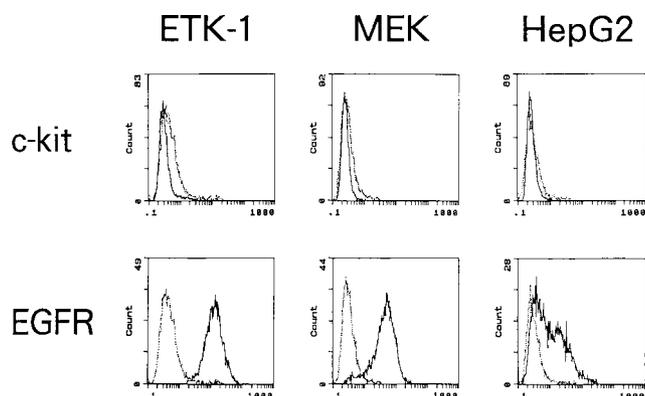


Fig. 2. Flow cytometric analysis for cell surface expression of c-Kit and the EGFR. Data are presented as histograms of cell number (y axis) versus fluorescence intensity (log scale, x axis). Dotted curves represent negative controls. EGFR is present, but c-Kit is absent on the examined cell lines, ETK-1, MEK, and HepG2.

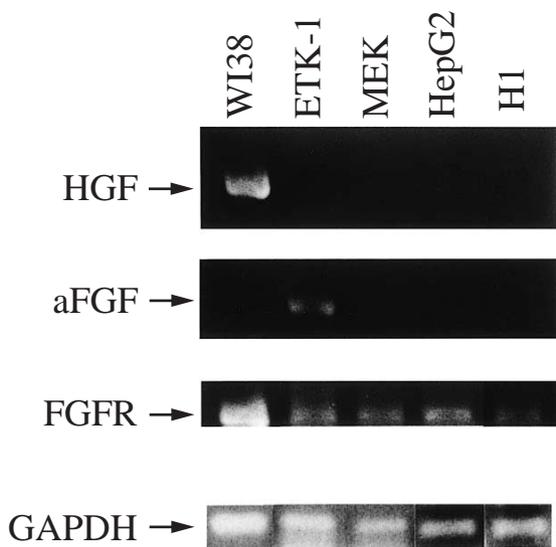


Fig. 3. Expression of HGF, aFGF, and the FGFR on ETK-1, MEK, HepG2, and H-1 cells, by RT-PCR. GAPDH expression was tested as a control. WI-38, a human fibroblast cell line, is the positive control for HGF.

EGF receptor, SCF receptor (c-Kit), HGF, aFGF, TGF α , and FGF receptor was tested by flow cytometric analysis (Fig. 2), RT-PCR (Fig. 3), or northern blot analysis (Fig. 4). All these cell lines revealed the same expression pattern for each of the markers, except for aFGF (Table III). c-Kit was not produced. c-Met was expressed but its ligand, HGF, was not. TGF α , aFGF, and their receptors were demonstrated in ETK-1 cells. In other words, the

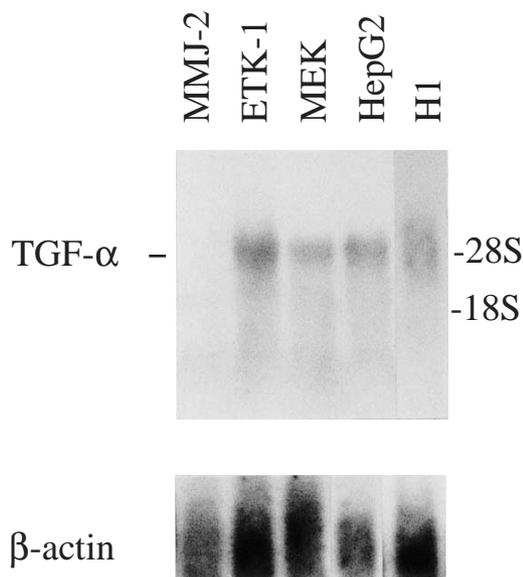


Fig. 4. Northern blot analysis for detection of the expression of the transcript encoding TGF α . The examined cell lines are shown at the top of the lanes. MMJ-2, a human melanoma cell line, is the negative control.

Table III. Expression of Cytokine/receptor Systems in Examined Cell Lines

Cytokine/receptor	ETK-1	MEK	HepG2	H-1
TGF α /EGFR	+/+	+/+	+/+	+/+
aFGF/FGFR	+/+	-/+	-/+	-/+
HGF/c-Met	-/+	-/+	-/+	-/+
SCF/c-Kit	NT/-	NT/-	NT/-	NT/-

TGF α , transforming growth factor- α ; EGFR, epidermal growth factor receptor; aFGF, acidic fibroblast growth factor; FGFR, FGF receptor; HGF, hepatocyte growth factor; SCF, stem cell factor; NT, not tested.

TGF α /EGF receptor and aFGF/FGF receptor systems can act on ETK-1 cells in an autocrine or a paracrine manner. **Effect of TGF α and aFGF** To ascertain whether TGF α or aFGF can affect ETK-1, MEK, H-1, or HepG2 cells, these factors were added to the culture media at various concentrations. There was no significant change in any of these cell lines after aFGF treatment (5–20 ng/ml). TGF α also induced no significant change in MEK, H-1, and HepG2 cells. However, ETK-1 cells revealed marked changes after TGF α stimulation. A few days after TGF α treatment, a number of large cells with extensive cytoplasm containing vacuoles of various sizes emerged (Fig. 5A and Table II) at every concentration of TGF α tested

(equal to or greater than 5 ng/ml of TGF α). Most of these morphologically transformed cells were intensely positive for the PAS reaction (Fig. 5B). This indicates that these cells are producing mucin. In addition, the mucinous substances were secreted into the culture medium (data not shown).

We examined immunocytochemically the expression pattern of marker proteins in TGF α -treated ETK-1 cells. The morphologically transformed cells revealed characteristics of bile duct cells (positivity for GGT and CK19), but hepatocytic markers (AFP and albumin) were absent (Table I). These phenotypic findings are similar to the parental cell line, ETK-1. However, in contrast to the original ETK-1 cells, CEA and CA19-9 were expressed, and vimentin was absent in the morphologically transformed ETK-1 cells (Table I and Fig. 5C). Quantitatively, 4–8% of cells showed obvious morphologic transformation into larger mucin-producing cells 48 h after TGF α -stimulation, and 20–40% of cells started to express CEA and CA19-9. These morphologic and phenotypic changes induced by TGF α treatment were significantly inhibited by pretreatment with 1 μ g/ml of anti-EGF receptor MoAb (Table II).

Expression of integrin molecules We examined the expression of integrins on ETK-1 cells with or without a 48-h incubation with TGF α (10 ng/ml), by flow cytometric analysis (Fig. 6). The treated cells were designated as ETK (+TGF α). Integrins α_2 , α_3 , and α_6 showed differences in expression between these two kinds of cells. The expression of these integrins on ETK-1 cells is evidently upregulated after TGF α stimulation.

Proliferative effect of TGF α The effect of TGF α on the proliferation of ETK-1 and MEK cells was also examined 72 h after stimulation (Fig. 7). The proliferation rate of MEK cells, which possess the properties of hepatocytes, increased in a dose-dependent manner after treatment with the factor. The proliferation rate of ETK-1 cells was significantly suppressed by TGF α .

DISCUSSION

Growth factors occasionally can induce differentiation of some types of cells.²⁴⁾ HGF,^{25, 26)} TGF α ,^{27, 28)} SCF,²⁹⁾ and aFGF³⁰⁾ work as growth-activators for hepatocytes. Therefore, we supposed them to be possible candidates as differ-

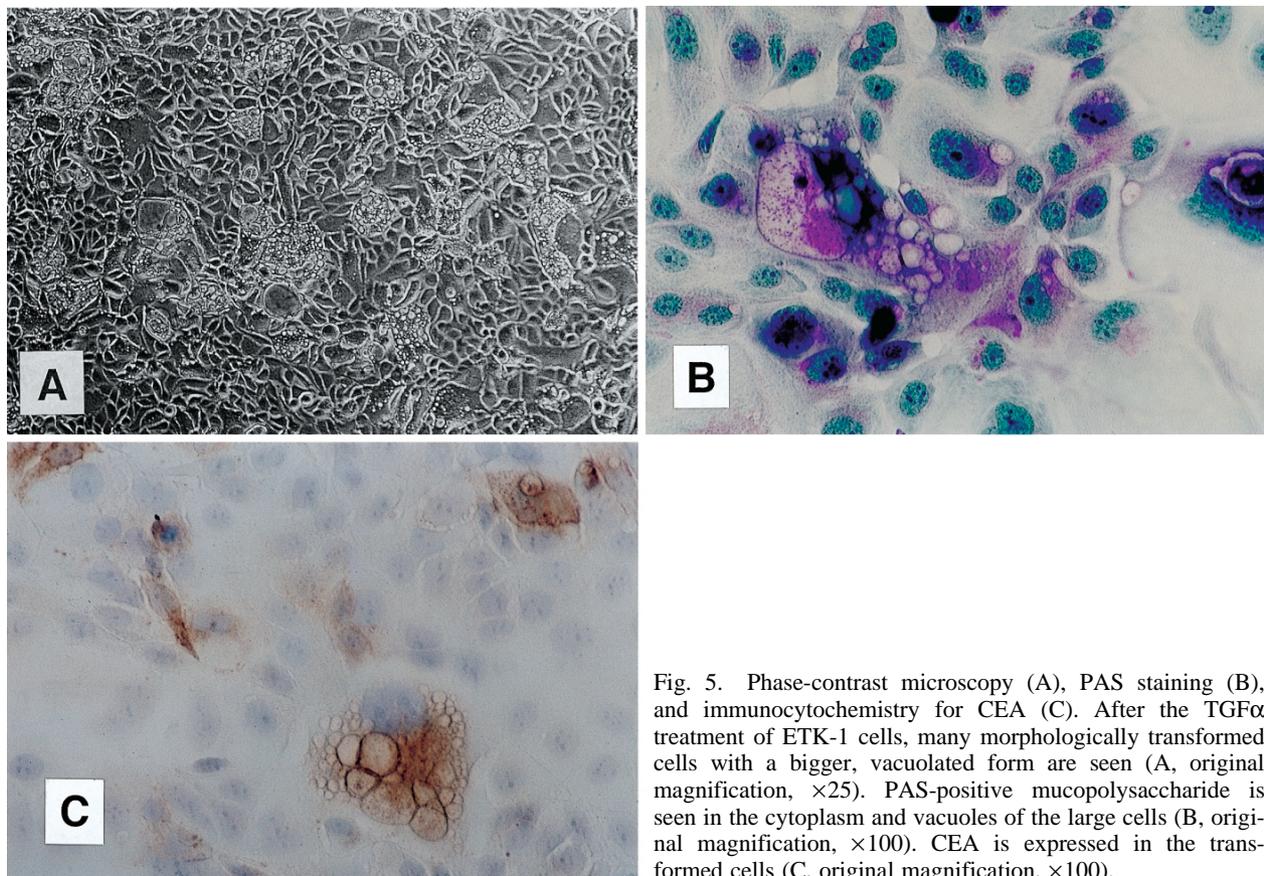


Fig. 5. Phase-contrast microscopy (A), PAS staining (B), and immunocytochemistry for CEA (C). After the TGF α treatment of ETK-1 cells, many morphologically transformed cells with a bigger, vacuolated form are seen (A, original magnification, $\times 25$). PAS-positive mucopolysaccharide is seen in the cytoplasm and vacuoles of the large cells (B, original magnification, $\times 100$). CEA is expressed in the transformed cells (C, original magnification, $\times 100$).

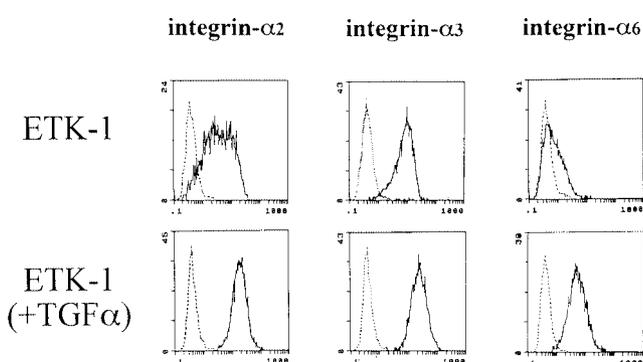


Fig. 6. Flow cytometric analysis of integrin subunit expression in ETK-1 cells with or without a 48 h exposure to TGFα. Data are presented as histograms of cell number (y axis) versus fluorescence intensity (log scale, x axis). Dotted curves represent negative controls.

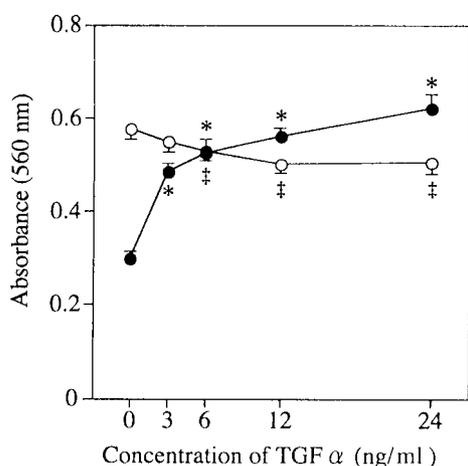


Fig. 7. Cell proliferation assay. The effect of TGFα on the proliferation of MEK and ETK-1 cells is shown. In this assay, cell number was estimated in terms of reduction activity for a tetrazolium compound after 72 h TGFα stimulation. All data are expressed as mean±SD and compared by one-way ANOVA in each experiment. Significance was evaluated with the Scheffe *F*-test and defined at the 1% level. Bars indicate SD. ○ ETK-1, ● MEK. * and †, significantly different from untreated (0 ng/ml) cells.

entiation-inducers and examined their effects on ETK-1 cells. It has not been clear whether TGFα can act as a differentiation factor in the liver. In this study, we showed that TGFα induced morphologic and phenotypic conversion of a sarcomatoid CC cell line, ETK-1. We consider these converted cells to have the characteristics of more differentiated CC cells because of the following facts: 1)

CEA and CA19-9 were newly expressed in the cells (Fig. 5C). The primary tumors in the patients' livers, from which ETK-1 cell line originated, contained well-differentiated and sarcomatous CC areas, and CEA and CA19-9 were expressed only in the adenocarcinomatoid cells, but not in the sarcomatoid cells.^{17,21)} 2) Vimentin, which is regarded as one of the marker proteins of immature traits,³¹⁾ disappeared in these converted cells. 3) The converted cells were larger and accumulated mucinous substances in their cytoplasm (Fig. 5, A and B). A larger cell size is preferentially seen in maturer cells of bile duct lineage, and mucin production is an important marker for identifying bile ductular cells or peribiliary glandular cells; maturer cells show this feature more clearly.³²⁾ 4) The converted cells did not proliferate as actively as ETK-1 cells, and the converted cell/ETK-1 ratio decreased when TGFα was removed from the culture medium (data not shown). This indicates that ETK-1 cells decrease their proliferative activity upon maturation. 5) All these changes induced by TGFα were significantly inhibited by anti-EGF receptor MoAb (Table II).

Based on these observations, we concluded that TGFα induces the differentiation of ETK-1 cells along a biliary epithelial lineage. In normal liver tissue, integrins α₂, α₃, and α₆ are exclusively expressed on/in bile duct epithelium, and are absent on/in other cell components without a vascular endothelium.³³⁾ Also, in CC tissue, the distribution pattern of integrins reflects the differentiation stage. Volpes *et al.* have reported that well-differentiated CC cells express integrins α₂, α₃, and α₆ completely, but undifferentiated CC cells show variable staining by antibodies against these molecules.³⁴⁾ After TGFα stimulation, more integrins α₂, α₃, and α₆ were expressed on the surface of the ETK-1 cells (Fig. 6). These findings also support this conclusion.

Evarts *et al.*³⁵⁾ and Alison *et al.*³⁶⁾ also have suggested the possibility that TGFα acts as a differentiation factor or a morphogen. Using rats exposed to 2-acetylaminofluorene and then partially hepatectomized, they demonstrated very strong TGFα-staining of newly forming bile ducts, which were the progeny of oval cells, and hypothesized that TGFα affects differentiation along the bile duct lineage, as well as tubule formation. Our data support and reinforce this hypothesis.

We tested the reaction of another sarcomatoid CC cell line, SSP-25, to TGFα (data not shown). The SSP-25 cell line was established from the spindle-shaped sarcomatoid component of CC.¹⁸⁾ SSP-25 cells are also spindle-shaped in culture and are positive for vimentin, but negative for CEA. The expression pattern of the growth factor/receptor systems was the same on SSP-25 cells as on ETK-1 cells. After TGFα treatment, the same changes occurred in SSP-25 cells as in ETK-1 cells. Many CEA- and mucin-positive, large cells with cytoplasmic vacuoles emerged.

ETK-1 cells were able spontaneously to change their morphology and phenotype, similar to cells converted by TGF α treatment, although the number was much smaller. This observation suggests that ETK-1 cells spontaneously differentiate into a maturer stage by producing TGF α themselves (Table III and Fig. 4). Why do untreated ETK-1 cells show a much lower efficiency for transformation than TGF α treated cells, despite the production of intrinsic TGF α ? It may be a dose effect of the exogenously added TGF α protein, because TGF α concentration was not measurable in culture medium of ETK-1 by our assay (data not shown). Another possible reason is qualitative difference between exogenously added TGF α and the intrinsic TGF α . Exogenously added TGF α is the smallest mature form and is 50 amino acids long. Intrinsic TGF α includes larger, incompletely processed forms and the membrane-anchored form.^{8, 15} Immature TGF α may have less activity than the mature form.

TGF α transgenic and knockout mouse lines were established in some laboratories and analyzed in detail. In transgenic mice, there was no unusual change of non-parenchymal cells in the livers and no stem-like cells (oval cells) were detected.³⁷ This observation coincides with our

results because, in our study, TGF α seemed to act on biliary epithelial cells as a differentiation factor, rather than a mitogenic factor. In knockout mice, liver tissue did not appear altered in structure or function.³⁸ This may be because some other cytokines, like EGF, act as proxies for TGF α , and it is suggested that TGF α is not indispensable for the differentiation of biliary epithelial cells.

We have demonstrated that 5-azacytidine-treated ETK-1 cells have the potential to differentiate into hepatocytes.¹⁷ In the present study, we showed that ETK-1 cells can be induced to differentiate along a biliary epithelial lineage by TGF α . These findings support our interpretation that ETK-1 cells possess bipotential differentiating activity.

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