Effects of Sodium Butyrate and Acidic Fibroblast Growth Factor on TDC32300 Cultured Cells

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Abstract

Background : Many studies have demonstrated that transition duct cells (TDC) are facultative liver stem cells. Our laboratory established TDC32300 cell lines with hepatic progenitor markers. The authors proposed that cell culture using sodium butyrate (NaBut) and acidic fibroblast growth factors (aFGF) may support the differentiation of TDC32300 cells along the hepatic lineage.

Methods : TDC32300 cells were cultured in four different conditions 1) STON media alone; 2) STON with NaBut in 3 different concentrations, 1 mM, 3.75 mM and 5 mM; 3) STON with aFGF; and 4) STON with aFGF and dexamethasone. After day 5, the cultured cells were fixed and stained with monoclonal antibodies to rat liver antigens and anti-proliferating nuclear antigen (PCNA).

Results : Proliferation of TDC32300 cells cultured in the high concentration of NaBut (3.75 and 5 mM) was inhibited. This phenomenon was confirmed by the reduction in cell number and decrease in PCNA expression. Irrespective of the concentration, NaBut did not alter the phenotype of the TDC32300 cultured cells. aFGF with or without dexamethasone also did not alter the phenotypic characteristic of TDC32300 cells.

Conclusion : TDC32300 cells may not be the hepatic progenitors or that their differentiation may require other culture conditions.

Key word : Sodium Butyrate, Acidic Fibroblast Growth Factor, Transitional Duct Cell, Facultative Liver Stem Cell

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The proliferation of small ductal-like cells, called oval cells, occurs when severe hepatic injury impairs the replicative capacity of normal hepatocytes. The oval cell population contains stem-like cells which can differentiate along hepatocytic, biliary, intestinal and pancreatic lineages in various rodent model systems[1-8]. Because activation of the oval cell compartment only occurs following severe liver injury, it has been proposed that oval cells are the progeny of facultative liver stem cells (FLSC).

Although the unequivocal origin(s) of FLSC in the normal adult liver remains unknown, Sell has proposed that the transition duct cell (TDC), which shares tight junctions with both a neighboring hepatocyte and another terminal biliary cell, are FLSC[6,7]. Identification, isolation and characterization of FLSC have been hampered by the lack of unique markers that unequivocally delineate this rare cell population of normal adult liver. Evidence that transition duct cells are FLSC was founded on the recent observations of Bisgaard et al[9]. These investigators showed that low levels of exposure to the liver carcinogen, 2-acetylaminofluorene, elicited a mitogenic response in a subpopulation of biliary epithelial cells and nondescript periductal cells within 24 hours after administration. Since the proliferative ductal cell population began to express hepatocyte transcription factors and genes, these investigators proposed that mitogenic ductal cells are FLSC[9]. The authors reasoned that if transition duct cells are FLSC then exposure to liver carcinogens will also lead to the active proliferation of this rare cell compartment which could be monitored by bromodeoxyuridine incorporation into newly synthesized DNA. Based upon the proposed rationale, our laboratory recently carried out a series of experiments to identify, isolate and characterize TDC from normal and carcinogen treated adult rat liver. The authors demonstrated that 1) TDC retains a developmentally immature ductal phenotype (eg. a phenotype expressed by newly forming bile ducts during fetal liver development); and 2) following exposure to a liver carcinogen, this cell population rapidly enters the cell cycle (unpublished observation). These findings suggested that TDC may be FLSC.

Our laboratory recently established several clonal hepatic epithelial cell lines from TDC. Preliminary data using reverse-transcriptase polymerase chain reaction (RT-PCR) analysis demonstrated that one of these cell lines designated TDC32300 contains transcripts for albumin and alpha-fetoprotein (AFP), suggesting that TDC32300 are hepatic progenitors (unpublished observation).

A major goal of our laboratory is to identify culture conditions that support the continued differentiation of TDC along the hepatocytic lineage. In order to address this question, the goal of this research project was to evaluate the effects of sodium butyrate (NaBut) and acidic fibroblastic growth factor (aFGF) on the differentiation potential of cultured TDC32300. The rationale for choosing these two differentiation agents is described below. The effects of NaBut and aFGF on the phenotype of TDC32300 cells was examined using monoclonal antibodies (MAbs) directed against cell type specific rat liver antigens (Table 1).

RATIONALE

Differentiation promotion by sodium butyrate and acidic fibroblast growth factor (aFGF)

Although, the mechanism of action of NaBut on cell differentiation *in vitro* is unclear, its effects are believed to be mediated through regulation of gene expression and as histone deacetylase inhibitor. The differentiating effects of NaBut may be related to its ability to block entry into the S phase, thus, inhibiting cell proliferation[10-12]. It should be noted that the effects of NaBut may depend on the concentration of this differentiation agent. For example, Gladhaug et al, demonstrated that when rat hepatocytes were cultured in the presence of 1 mM NaBut DNA synthesis was slightly elevated; however, DNA synthesis was inhibited at 5 mM concentration[10].

Sodium butyrate has been shown to be a potent modulator of the *in vitro* differentiation of oval cells in primary culture. Differentiation is the process of acquiring individual characteristics as occurs in the diversification of embryonic cells and tissues. In primary culture of AFP positive, albumin positive oval cells, NaBut (3.75 mM) induced a massive production of albumin and induced tyrosine aminotransferase activity while inhibiting DNA synthesis[13]. In contrast, another study of Germain et al showed that when early fetal rat hepatoblasts were exposed to NaBut (3.75 mM) these bipotential hepatic progenitors differentiated along the biliary lineage[1]. Based on these data, the authors hypothesized that if TDC32300 cells are FLSC then exposure to NaBut will lead to the expression of hepatic and/or biliary markers.

Jung et al, have shown that treatment of isolated foregut endoderm from mouse embryos with fibroblast growth factors (FGF1, FGF2) was suffi-

Table 1. Summary of MAbs to rat liver cells generated by various investigators using different immunization techniques[17].

Mab designation	Antigen designation	Antigen identity	Oval cells	Bile ducts	Hepatocytes	Hematopoietic cells
270.38	OC.2	cell surface	positive	positive	negative	negative
OV.6	OV.6	cytoplasmic	positive	positive	negative	negative
5.4	Cell-CAM 105	cell adhesion molecule	negative	negative	positive	negative
368.7	H.4	cytoplasmic	negative	negative	positive	negative
282.16	DP.1	desmoplakin 1	negative	positive	positive	negative
Albumin	Albumin	albumin	positive	negative	positive	negative
AFP	AFP	AFP	positive	positive	positive	negative
Con 43	Connexin 43	Connexin 43	positive	positive	negative	negative
OX 7	Thy 1	Thy1	positive	negative	negative	positive
Con 32	Connexin 32	Connexin 32	negative	negative	positive	negative

cient to replace cardiac mesoderm as an inducer of the liver gene expression program. The hepatogenic response was restricted to endoderm tissue, which selectively co-expresses FGF receptors 1 and 4[14]. Further studies with FGF and their specific inhibitors showed that FGF8 contributes to the morphogenetic growth of the hepatic endoderm. Thus, different FGF signals appear to initiate district phases of liver development during mammalian organogenesis. Spagnoli et al, recently demonstrated that exposure to acidic FGF (i.e. FGF1) was required to initiate the hepatic differentiation program of palmate cells. Palmate cells are hepatic progenitors derived from the fetal liver of transgenic mice over expressing the c-met receptor[15]. Therefore, the effects of aFGF on the phenotypic plasticity of TDC32300 cells were also examined.

MATERIAL AND METHOD

Material

n-Butyric acid sodium salt was obtained from Sigma Chemical Co., St. Louis, Missouri. Lab-Tek Chamber slides were obtained from Fisher Scientific, Houston, Texas. Goat anti-mouse polyvalent-FITC antibodies were obtained from Sigma Chemical Co., St. Louis, Missouri. Texas Red Goat anti-mouse IgM antibodies were obtained from Vector Laboratories, Burlingame, California. Rabbit anti-Sheep IgG FITC antibodies were obtained from Pierce, Rockford, Illinois. Anti-Proliferating Cell Nuclear Antigen (PCNA) was obtained from Boehringer Mannheim, Indianapolis, Indiana. Streptavidin Texas Red antibodies were obtained from Molecular Probes, Eugene, Oregon. Fetal bovine serum (FBS) and normal goat serum (NGS) were obtained from Sigma Chemical Co., St. Louis, Missouri. Dulbecco's modified Eagle's medium was from Gibco, Grand Island, New York. Acidic fibroblast growth factor (aFGF) was obtained from Gibco, Grand Island, New York.

TDC32300 cells for culture were prepared from early passage cell lines (passage 8+) propagated in T-75 polystyrene tissue culture flasks and cell line specific media (STON). Cells were split at confluency with trypsin/EDTA (trypsin was neutralized with FBS) and suspended in media (STON) on ice until use.

Method

Cell culture

TDC32300 cells were seeded on Lab-tek Chamber slides (100,000 cells/well) in STON media (Dulbecco's modified Eagle's medium containing 10 per cent fetal bovine serum, 1 per cent non-essential amino acid, 50 ug/ml gentamicin and 0.0025 µl/ml β-mercaptoethanol). Cells were allowed to attach at 37°C for approximately 4 hours. The plating media was replaced by STON media containing sodium butyrate at 0, 1, 3.75 and 5 mM concentrations. Cells were refed with the same media on days 3 and 5.

In one experiment, TDC32300 cells were cultured with aFGF (100 ng/ml) and heparin (10 µg/ml) with and without dexamethasone (1.96 µl/ml).

Monoclonal antibodies to rat liver antigens

MAbs have been developed against rat liver oval cell, bile ductular cell, and adult hepatocyte antigenic determinants. Although the identity of most of these antigens are unclear the MAbs to these anti-

gens are a valuable tool for the isolation and defining the lineage phenotype of rat liver cells (Table 1). It should be noted that it was recently reported that the hematopoietic marker, Thy 1, is expressed by oval cells[16].

Anti-proliferating cell nuclear antigen (PCNA) staining

PCNA begins to accumulate during the G1 phase of cell cycle, is most abundant during the S phase, and declines during the G2/M phase. It is a marker for cell proliferation. PCNA expression can be detected immunochemically using a commercially available monoclonal antibody. Since the differentiation of TDC cells may only occur if NaBut inhibits their proliferation, the effects of this differentiation agent on PCNA expression was monitored as described in the instructions from the supplier.

Indirect immunofluorescence staining

After day 5, cultured cells were washed with PBS and fixed with methanol for PCNA and albumin immunostaining, and with acetone for all other antibodies. The immunostaining protocol was performed as described by Faris et al[17].

RESULTS

Effects of NaBut on cell morphology and proliferation

As shown in Fig. 1, exposure to the higher concentrations of NaBut (3.75 and 5.0 mM) dramatically inhibited the proliferation of the spindle-shaped

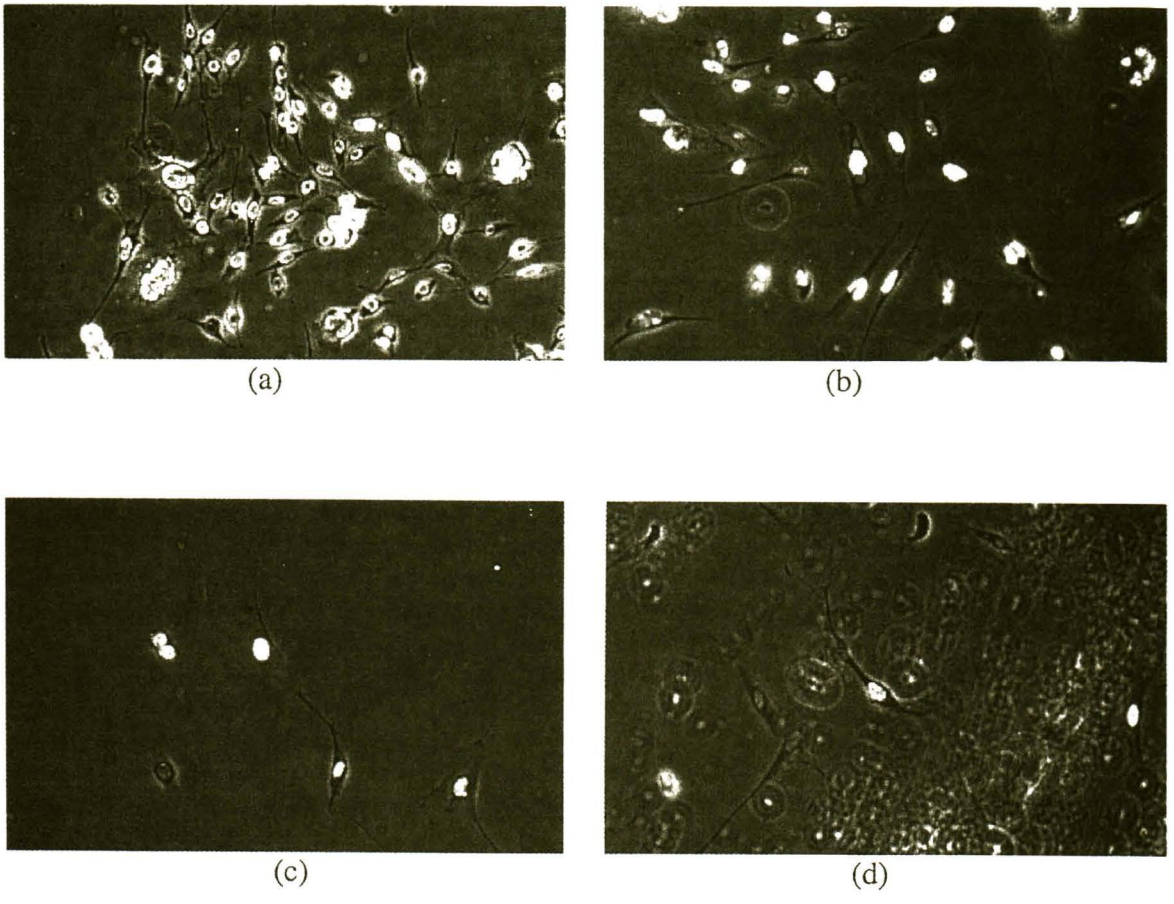

Fig 1. PCNA analysis of the cultured TDC32300 cells after day 5. (a) cells in STON media, (b) cells in 1 mM NaBut, (c) cells in 3.75 NaBut, (d) cells in 5 mM Nabut.

Table 2. Immunophenotyping of cultured HSC 32300 cells in different media.

MAbs	STON	1 mM NaBut	3.75 mM NaBut	5 mM NaBut	aFGF with dexamethasone	aFGF
AFP	+	+	+	+	ND	ND
ALB	-	-	-	-	-	-
OC2	-	-	-	-	-	-
DP1	-	-	-	-	-	-
5.4	-	-	-	-	+(weak/het)	-
Con 32	-	-	-	-	-	-
368.7	-	-	-	-	-	-
OX 7	-	-	-	-	+(weak/het)	+(weak/het)
OV6	-	-	-	-	-	-
Con 43	-	-	-	-	ND	ND
PCNA	+	+	+(weak/het)	+(weak/het)	ND	ND

\+ = >95% of cells composing confluent cultures positive with staining intensity >1+/4+
\- = not detected
weak = staining intensity 1+/4+
het = 5-95% of cells composing confluent cultures positive
ND = not determined

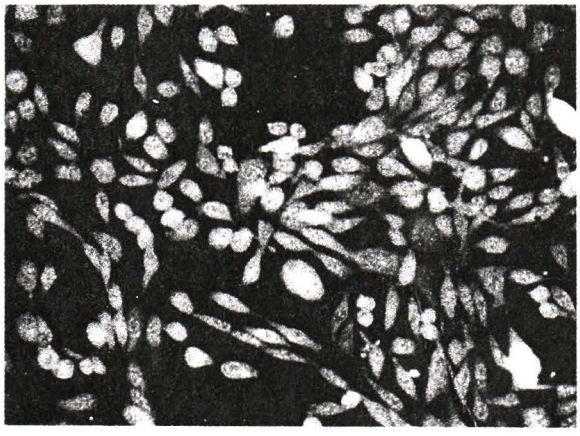

Fig. 2. AFP positive TDC32300 cultured cells in STON media after day 5.

TDC cells, as indicated by the reduction in cell number and the decrease in PCNA expression. In the higher concentrations of NaBut, cells were weakly and heterogeneously positive and assumed a more fibroblastic appearance.

Phenotypic characterization of cultured TDC32300 cells in NaBut or aFGF using rat liver MAbs

Irrespective of the concentration, NaBut did not alter the phenotype of TDC32300 cultured cells (Table 2). Immunostaining of TDC32300 cells demonstrated that these cells expressed high levels of AFP, a marker of hepatic progenitor cells (Fig. 2). NaBut exposure did not further induce any hepatocyte or biliary specific markers. This finding suggested that TDC32300 cells may require exposure to additional promoter(s) in order to differentiate. Therefore, the effects of aFGF, in the presence of heparin with and without dexamethasone, on the phenotype of TDC32300 cells were examined. The authors found that aFGF did not significantly alter the phenotype of TDC32300 cells under any culture conditions.

DISCUSSION

The authors hypothesized that TDC32300 cells were bipotential hepatic progenitor cells derived from the normal adult liver. This reasoning was based upon the previous demonstration that these cells express transcripts for AFP and albumin, markers of bipotential hepatoblasts. The purpose of this project was to determine if either NaBut or aFGF could induce the differentiation of TDC along either the hepatocytic or biliary lineages. Both of these agents have previously been used by other investigators to induce the hepatic differentiation of progenitor cells[10-14].

Since it has been proposed that replicating cells will not differentiate, the authors examined the effects of different concentrations of NaBut on the proliferation and phenotype of TDC32300 cells. In

the present study, exposure to NaBut, did not alter the phenotype of TDC32300 cells even though the higher concentrations of NaBut (3.57 mM and 5 mM), significantly inhibited their proliferation as measured by cell number and the expression of PCNA. It should be noted that the present findings suggested that the NaBut actually prolonged the duration of the cell cycle since even at 5.0 mM NaBut, some cells weakly expressed PCNA. The authors subsequently postulated that TDC32300 cells lines may need another promoter such as acidic FGF in order to differentiate.

The experiment was repeated using aFGF to demonstrate whether TDC32300 cultured cells in the presence of aFGF with or without dexamethasone were induced to express either hepatogenic or biliary phenotype. The present results have demonstrated that aFGF did not alter phenotypic characteristic of TDC32300 cells. Although, TDC32300 cultured cells contain transcript for albumin and α-fetoprotein (our preliminary data using RT-PCR analysis) which were found in hepatic progenitor cells, these cells were unable to differentiate in both NaBut and aFGF. This suggested that TDC32300 cells were not hepatic progenitors or that their differentiation may require other culture conditions such as an appropriate matrix. Alternatively, the ability of TDC to respond to hepatic differentiating agents may be impaired once these cells were removed from their native microenvironment. In the future this question may be addressed by performing hepatic transplantation experiments.

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ผลของ sodium butyrate และ acidic fibroblast growth factor กับการเจริญเติบโตของเซลล์เพาะเลี้ยง TDC32300

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Transitional duct cells (TDC) เป็น facultative liver stem cells (FLSC) ซึ่งทางห้องทดลองของคณะ ผู้วิจัยที่เพาะเลี้ยงจาก cell lines ซึ่งมีต้นกำเนิดจากเซลล์ตับของหนู โดย cell lines นี้มีคุณสมบัติของ hepatocyte เราตั้งชื่อ cell lines กลุ่มนี้ว่า TDC32300 คณะผู้วิจัยได้ศึกษาถึงการเพาะเลี้ยงเซลล์กลุ่มนี้ในสภาวะแวดล้อมต่าง ๆ กัน เพื่อจะดูว่าเซลล์กลุ่มนี้มีการเปลี่ยนแปลงเจริญไปเป็นกลุ่มเซลล์ทางด้าน hepatocyte หรือกลุ่ม cholangiocyte หรือทั้งสองอย่าง การเพาะเลี้ยงกลุ่ม cell TDC32300 นี้ได้แบ่งออกเป็น 6 สภาวะแวดล้อม โดยการเติมสารต่าง ๆ ดังต่อไปนี้ กลุ่ม 1) ใช้แต่ media 2) เติม sodium butyrate (NaBut) 1 mM 3) เติม NaBut 3.75 mM 4) เติม NaBut 5 mM 5) เติม acidic fibroblast growth factor (aFGF) 6) เติม aFGF และ dexamethasone

หลังการเพาะเลี้ยง 5 วัน ก็จะนำ TDC32300 มาย้อมด้วย monoclonal antibody ต่อเซลล์ตับของหนู และย้อมด้วย anti-proliferating nuclear antigen (PCNA) เพื่อดูการแบ่งตัวของเซลล์

ผลการศึกษาพบว่าเซลล์ TDC32300 ที่เลี้ยงด้วย NaBut ในปริมาณ 3.75 และ 5 mM มีการยับยั้งการแบ่งตัวของเซลล์กลุ่มนี้ และจากการย้อมด้วย PCNA ก็พบว่ามี expression หรือแบ่งตัวลดลง NaBut ในปริมาณต่าง ๆ, aFGF หรือ aFGF + dexamethasone นั้น ไม่มีผลต่อการเปลี่ยนแปลงทาง phenotype ของเซลล์กลุ่มนี้ ดังนั้นจึงอาจเป็นไปได้ที่ TDC32300 ไม่ใช่ hepatic progenitors หรือการแบ่งตัวเพื่อนำไปสู่การเปลี่ยนแปลง อาจต้องการสารเพาะเลี้ยงหรือสภาวะแวดล้อมแบบอื่น

คำสำคัญ : โซเดียม บิวไทเรต, อะซิดิก ไฟโบรบลาส โกรธแฟคเตอร์, ทรานสิชั่นนัล ดักท์เซลล์, ฟิลคัลเททีฟ สเตมเซลล์ของตับ

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