Generation and characterization of immortalized rat pancreatic stellate cells

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mouse IgG, R-phycoerythrin-streptavidin from MoBiTec (Göttingen, Germany); PDGF-BB, recombinant transforming growth factor-β1 (TGF-β1) from R&D (Wiesbaden, Germany); 15-deoxy-Δ12,14-prostaglandin J2 (PGJ2) from Calbiochem (Schwalbach, Germany); RNeasy Mini RNA extraction kit, Taq polymerase from Qiagen (Hilden, Germany); Nycodenz from Nycodent (Oslo, Norway); [2,3,3H]proline, horseradish peroxidase-labeled anti-mouse Ig, and ECL plus kit from Amersham (Freiburg, Germany); hygromycin B from Clontech (Heidelberg, Germany); rat-tail collagen from Tebu (Frankfurt, Germany); primers for PCR were generated by using the NCBI genebank as the source for any sequences and were synthesized by BioTez (Berlin, Germany); rat genome U34 microarray, biotin-11-CTP, biotin-16-UTP, and BioArray HighYield RNA transcript labeling kit from Affymetrix (Santa Clara, CA); and biotinylated anti-streptavidin antibody from Alexis (Grünberg, Germany).

Male LEW.1W inbred rats were obtained from the University of Greifswald, Germany. All studies were approved by the local animal ethics committee (ref. no. 7221.3-2.3.1-001).

Isolation and culture of PSCs. Stellate cells were isolated from the pancreas of male LEW.1W inbred rats, as previously described (16). Briefly, the common pancreatic-bile duct of anesthetized and exsanguinated rats was cannulated followed by intraduodenal instillation of 8 ml of HBBS containing 0.05% collagenase P, 0.02% protease IX, and 50 μg/ml DNase. The distended pancreas was removed, minced, and shaken in the enzyme-containing HBBS. After a further dispersion achieved by up-and-down pipetting, the tissue was centrifuged and the pellet was resuspended with HBBS containing 4% FCS. The cell suspension was filtered through a 150-μm cell sieve, centrifuged, and resuspended. It was then mixed with a stock of the gradient medium Nycodenz to obtain a final concentration of 12% Nycodenz. The cell-Nycodenz suspension was layered underneath 6 ml of HBSS and centrifuged for 20 min at 1400 g. The band just above the Nycodenz cushion containing the cells of interest was harvested, washed, and resuspended in IMDM supplemented with 17% FCS, 0.1 mM NEA, and antibiotics (penicillin 100 units/ml, streptomycin 100 μg/ml). Isolated cells were left to adhere to tissue culture plates. After reaching confluence, the cells were passaged by trypsinization according to standard protocols. Incubation of cells for proliferation assays and for the determination of collagen production was performed in serum-free HE medium.

Construction of the SV40 large T antigen encoding retroviral vector. A 2.5-kb BamHI fragment encoding a temperature-sensitive mutant of the SV40 large T antigen [LTSsAS8/U19; (21)] was inserted into a murine leukemia virus (MLV)-based retroviral vector [pLXSN; (26)] in front of an internal ribosomal entry site fused to a hygromycin resistance encoding gene. The complete cassette (SV40LT/IRES/hygR) is flanked by loxp sites enabling excision of the immortalizing gene by Cre recombinase. This would facilitate expression of a downstream-located neomycin resistance gene via the 5’ LTR. The generated vector pLLTHN3 (Fig. 1) was transfected into 293 (human embryonic kidney ATCC CRL-1573)-based packaging cells stably transfected with a MLV Gag-Pol encoding plasmid [pGag-PolGpt, (23)] and pALF (8) carrying the MLV Env encoding region. Stable producer cells were selected at 37°C by the addition of hygromycin (100 μg/ml). Infection of mouse NIH3T3 cells followed by hygromycin selection revealed virus titers of 3 × 107/106 cells/ml.

Retroviral infection and immortalization of rat PSCs. PSCs on days 7 and 14 after isolation from the rat pancreas were used for immortalization. For this approach, virus producer cells were grown to 80% confluence in a T75 flask and cultivated overnight with 4 ml of DMEM containing 10% FCS at 37°C, 5% CO2, 95% humidity. Virus-containing supernatant was filtered (0.2 μm) and 1 ml was applied to primary cells grown in T25 flasks. After 6 h, infected cells were replenished with 4 ml of DMEM/15% FCS supplemented with nonessential amino acids and insulin (7 μg/ml) and shifted to the permissive temperature (33°C). After 4 days in culture, cells were selected for stably integrated LLTIHN3 by adding hygromycin in increasing concentrations (50 μg/ml to 100 μg/ml). After 2–5 wk, stable populations were obtained, expanded, and further analyzed.

Immunofluorescence. Cells were cultivated on glass coverslips. For immunofluorescence staining, cells were fixed with ice-cold methanol followed by incubation with a mouse mAB-detecting vimentin and SMA. Binding of specific mAB was determined by a fluorescein-labeled goat anti-mouse IgG and visualized by using a fluorescence microscope.

Histochemical staining of intracellular fat. Intracellular fat droplets were visualized by Oil Red O staining. On glass coverslips, plated cells were fixed in 2.5% paraformaldehyde for 30 min, incubated with Oil Red O (1% wt/vol dissolved in isopropanol) for 15 min and counterstained with Mayer’s hematoxylin. Treatment with PGJ2 was performed for 72 h using the standard cell culture conditions.

Microarray analysis of RNA expression profiles. To obtain a comprehensive overview of the RNA expression profiles and indications of differentially expressed genes, we used the microarray technique comparing RNA levels in PSCs and the LTC lines. The objective of these experiments was to determine whether the immortalized cell lines can be used as a suitable PSC model with respect to genes whose expression is considered to be substantially involved in fibrogenesis. The high-density oligonucleotide array hybridization was performed according to the supplier’s instructions. Briefly, total RNA from the respective cells was extracted by using the RNeasy kit. First-strand cDNA synthesis was carried out by superscript II reverse transcriptase and T7-dT25 primer using 10 μg of RNA. Second-strand synthesis was done according to the SuperScript Choice system followed by an in vitro transcription reaction to label the cRNA samples with biotin-11-CTP and biotin-16-UTP. The labeled cRNA was hybridized to the chips overnight at 45°C. Staining was performed with R-phycoerythrin-streptavidin followed by an antibody...
amplification procedure using a biotinylated antistreptavidin antibody and goat IgG. The fluorescence intensity was scanned, and data were scaled on the basis of total intensity with the Affymetrix array software.

Analysis of gene expression was carried out by means of the Rat Genome U34 Array interrogating 7,500 genes. To ensure that the data-analyzing process will only perform valid expression data, we used a software tool developed in Visual Basic Application for Excel. This tool performs a filter algorithm for microarray expression data using validity rules to check the consistency of different parameter values of each gene.

In this study, we compared the expression profiles of primary PSCs after 14 days in culture with the LTC-14 line using a single array for each. Transcript levels in LTCs were defined as baseline, and results were given as fold change of the mRNA expression in the respective PSCs. To consider only valid data of similar expression levels in LTCs and PSCs, the stronger cutoff of $\approx 4$ was taken.

RT-PCR. Total RNA was isolated from cells using the RNeasy kit according to the manufacturer’s instructions. One microgram of total RNA was reverse transcribed into cDNA as described by Sparmann et al. (33) using oligo(dT)$_{12-18}$ primer and superscript II reverse transcriptase. Quantification of mRNA expression was performed by using competitive PCR for the housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT). The respective cDNA samples were coamplified with a defined concentration of a synthetic DNA fragment as internal standard containing HPRT specific primer sequences. The molecular weight of the standard and cDNA amplicons were different, allowing electrophoretic separation in an 1.8% agarose gel containing 0.3 g/ml ethidium bromide. On the basis of the intensity of the ethidium bromide fluorescence that reflects the HPRT expression, the various cDNA samples were adjusted to equal input concentrations (33).

**Fig. 2.** Expression of the SV40 large T antigen (SV40 LT) protein detected by immunoblotting. Protein extracts of SV40 LT immortalized cell lines (LTC-7, LTC-14) as well as of pancreatic stellate cells (PSCs) as a negative control were harvested and electrophoretically separated on an 8% SDS polyacrylamide gel. After protein transfer onto a nitrocellulose membrane, blots were incubated with a mouse-monoclonal antibody detecting SV40 LT. Detection of primary antibody binding was performed by using a horseradish peroxidase-labeled rabbit anti-mouse IgG. Horseradish peroxidase activity was visualized with the ECL Plus kit. LTC-7 (lane 1) and LTC-14 (lane 2) immortalized by LT gene transfer were shown to express the SV40 LT protein (arrow), whereas primary PSCs were negative (lane 3).

**Fig. 3.** Immunocytochemical analysis of α-smooth muscle actin (SMA) and vimentin expression. Primary PSCs obtained from rat pancreatic tissue by collagenase digestion and density gradient centrifugation were used. For the immunohistochemical procedure, cells were transferred onto glass coverslips and grown by using standard culture conditions. Cell staining was performed by incubation with specific mouse monoclonal antibodies (mAb) detecting SMA and vimentin, respectively. Binding of primary antibody was visualized with a fluorescein-labeled goat anti-mouse IgG resulting in green fluorescence. Microphotographs of primary PSCs at day 1 (A and D), day 3 (B), and day 14 (C and E) after isolation demonstrating the expression of SMA (A–C) or vimentin (D and E) are shown. To demonstrate the specificity of the respective primary antibody, PSC-14 were incubated only with the fluorescein-labeled IgG as a control (F) (original magnification, $\times630$).
Cell proliferation assay. Cell proliferation was assessed by using the colorimetric BrdU DNA-incorporation assay. Therefore, cells suspended in IMDM with 17% FCS were seeded into 96-well plates (2 × 10^4 cells/well). Adherent cells were treated for 24 h with PDGF dissolved in serum-free HE medium. BrdU labeling of PSCs and LTCs was performed during a further 24 and 4 h incubation, respectively. The subsequent incubation steps were carried out according to the manufacturer’s instructions. Finally, the absorbance at 450 nm was recorded with a 96-well plate reader.

Immunoblotting. Western blot analysis was performed as described elsewhere (16). Briefly, cells were harvested by medium aspiration and the addition of ice-cold lysis buffer (in mM: 20 Tris-HCl, pH 7.5, 150 NaCl, 2 EDTA, 1 PMSF, 1 sodium orthovanadate, and 25 β-glycerophosphate with 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10% glycerol, and 0.15 U/ml aprotinin,) directly to the cell monolayer. Lysates were collected and centrifuged at 4°C for 5 min at 10,000 g. Fifteen micrograms of protein were separated by SDS-PAGE and blotted onto nitrocellulose filters. The membranes were then blocked with 1% BSA and incubated with the indicated protein-specific antibodies overnight at 4°C. After a final incubation with a horseradish peroxidase-labeled anti-mouse Ig antibody, the blots were developed by using the ECL Plus kit. The intensity of the resulting bands, reflecting the amount of the respective protein, was measured with an electronic camera analyzing the data with the EASY program (Herolab, Wiesloch, Germany). Results are expressed as relative density with respect to an external standard (means ± SE).

Measurement of collagen production. Collagen production by PSCs or LTCs was quantified by assessing the incorporation of [2,3-3H]proline as described by Becker et al. (5). Cells were seeded into 12-well plates. After reaching 90% confluence, cells were cultured for 24 h in the presence of 2.5 μCi/ml [2,3-3H]proline, 50 μg/ml ascorbate, 50 μg/ml BAPN, as well as the TGF-β1 or PGJ2 at indicated concentrations dissolved in serum-free HE medium. Forward, 50 μg/ml 10 N acetic acid was added to each well and the culture plates were left at 4°C overnight. For protein precipitation, cell supernatants were transferred into tubes and mixed with 100 μg/ml FCS, 5 μg/ml rat-tail collagen, and 250 μg/ml 25% NaCl dissolved in 0.5 N acetic acid. The mixture was incubated at 4°C for 30 min and centrifuged at 18,000 g for 30 min at 20°C. After the [3H]proline-containing pellet was washed with 5% NaCl, it was dissolved in 0.5 N acetic acid followed by evaluation using liquid scintillation counting. Data were calculated from triplicate determinations and expressed as counts·min⁻¹·well⁻¹. To correct the measured values for different cell numbers, the plates containing the remaining adherent PSCs or LTCs were stained with methylene blue, the adsorbed amount of which was proportional to the respective cell number. After fixation with methanol, cells were incubated with 0.1% methylene blue dissolved in 0.1 M sodium borate, pH 8.5, for 30 min. After cells were washed, the bound dye was dissolved in HCl/ethanol (20:80) and the color intensity was measured photometrically at 450 nm.

Statistical analysis. Results are expressed as means ± SE. Data were analyzed by using Wilcoxon’s rank sum test and P ≤ 0.05 was considered to be statistically significant.

RESULTS

Generation of immortalized rat stellate cells and immunohistochemical characterization. Infection of primary PSCs 7 and 14 days after isolation with a recombinant retroviral vector encoding the SV40 large T antigen followed by the selection with hygromycin B resulted in two stable cell lines (LTC-7 and LTC-14) expressing the SV40 large T antigen protein of ~85 kDa as revealed by Western blotting (Fig. 2).

The immunohistochemical staining of primary PSCs after 1 day in culture, demonstrated their stellate-like morphology lacking SMA expression (Fig. 3A). Figure 3 illustrates the typical transformation of primary PSCs during cultivation into a myofibroblastic cell type associated with the formation of SMA stress fibers (day 3, Fig. 3B; day 14, Fig. 3C). The cell line LTC-14 retained the characteristics of the 14 days in vitro-cultivated primary cells they were derived from in terms of morphology and SMA expression (Fig. 4B). In contrast, the LTC-7 line showed a more stellate-to-fibroblastic cell morphology without SMA stress fiber organization (Fig. 4A).

![Fig. 4. Immunocytochemical analysis of SMA and vimentin expression. Propagated immortalized cell lines were processed as described in Fig. 3. Microphotographs of LTC-7 (A and C) and of LTC-14 (B and D) stained for SMA (A and B) or vimentin (C and D) are shown. E and F: demonstration of the secondary antibody control for LTC-7 (E) and LTC-14 (F) (original magnification, ×630).](http://ajpgi.physiology.org/Downloadedfromhttp://ajpgi.physiology.org/)
In agreement with immunohistochemical data, SMA expression revealed by Western blotting (Fig. 5) was low in 24 h- and 3 days-cultivated PSCs (lanes 1 and 2), increased at day 5 (lane 3), and reached significantly higher levels after 2 wk (lane 4). Furthermore, the LTC-14 line and 14 days-cultivated PSCs expressed similar amounts of SMA, whereas LTC-7 showed a weak SMA-specific band.

In contrast to SMA, vimentin was regularly expressed in PSCs independently of the activation status and in both LTC lines as revealed by immunofluorescence staining (Figs. 3, D and E, and 4, C and D) and Western blotting (Fig. 5).

Immortalized cell lines could be maintained in culture for extended periods without becoming senescent or going into crisis. Whereas PSCs degenerated after repeated passages, LTCs continued to proliferate, to date >100 passages, without change in gross morphology or evidence of undergoing senescence in terms of proliferation rate, expression profile, or responsiveness to growth factors.

Cell proliferation. PDGF is a well-known potent mitogen for various cell types. The effect of PDGF on the DNA synthesis rate of primary stellate cell cultures and immortalized cell lines was determined by using the BrdU incorporation assay. Figure 6 shows the mean proliferation rate of primary cells in response to PDGF-BB compared with the immortalized lines. PDGF enhanced the cell growth in a concentration-dependent manner, and the growth factor-mediated stimulation had a similar course in all experiments, with a maximum effect for PSCs and LTCs at a PDGF concentration of 10 ng/ml. However, the relative increase of proliferation induced by PDGF was higher in PSCs compared with LTCs, due to a general, faster division rate of immortalized cells. The basal doubling time of the LTC-7 line was found to be 15.4 h ± 3.2 h (n = 10) and the LTC-14 cells reduplicated during 20.6 h ± 2.9 h (n = 10). In contrast to the constant growth of the cell lines, the proliferation rate of the primary PSCs was highly dependent on the time in culture and characterized by a great variability. Activation of isolated PSCs caused by the in vitro cultivation was characterized by an increase of cell proliferation reaching maximum values around the fourth day after isolation with a doubling time of 22 ± 7 h (n = 6). Thereafter, the cell division rate decreased consistently. The reduplication rates averaged 30 ± 6 h on day 7 and 45 ± 12 h on day 14.

Collagen production. PSCs and both LTC lines were found to produce and secrete collagen as determined by [3H]proline incorporation. Primary and immortalized cells responded to the profibrogenic cytokine TGF-β1 as well as to the PPARγ ligand

![Fig. 5](image)

Fig. 5. Western blot analysis of expression of SMA and vimentin in primary PSCs and immortalized cell lines. Cultured PSCs at day 1 (lane 1), day 3 (lane 2), day 5 (lane 3) and day 14 (lane 4) after isolation, LTC-7, or LTC-14 were harvested and, protein extracts were prepared. Results are shown as the means of relative density ± SE; *P = 0.05.

![Fig. 6](image)

Fig. 6. Influence of PDGF on cell proliferation. Primary PSCs, LTC-7, and LTC-14 suspended in Isocote’s modified Dulbecco medium (IMDM) supplemented with 17% FCS were seeded into 96-well culture plates (2 × 10³ cells/well) and then incubated for 24 h in serum-free medium containing PDGF at the indicated concentrations. After the addition of 5-bromo-2’-deoxyuridine (BrdU), PSCs were cultured for a further 24 h and LTCs for 4 h. As a control, cells were kept for the same time in serum-free medium without PDGF. Data are given as means ± SE; n = 6, *P ≤ 0.05 with regard to the respective control.

![Fig. 7](image)

Fig. 7. Influence of TGF-β1 and 15-deoxy-Delta12,14-prostaglandin J2 (PGJ2) on collagen production of PSCs and LTCs as determined by [3H]proline incorporation. Primary and immortalized cells responded to the profibrogenic cytokine TGF-β1 as well as to the PPARγ ligand...
PGJ2, TGF-β1 increased the collagen synthesis to a higher degree in PSCs compared with LTCs, whereas the inhibitory effect of PGJ2 was stronger with LTCs than with PSCs (Fig. 7). When LTC-7 and LTC-14 were compared, in each case, the response of LTC-14 was quantitatively more pronounced.

Expression profile. To obtain an overview of the RNA expression profiles in LTCs compared with the activated primary cells, we used the microarray technique. Here, we focused the data evaluation on genes that might be relevant for fibrogenesis and known markers of PSCs. Figure 8 shows selected data of a chip experiment investigating 14-day-old PSCs and LTC-14. Defining the fold change threshold at 4, there were no significant differences in the RNA levels of genes encoding components of the ECM (e.g., collagen type I and III, fibronectin, laminin, and tenasin), integrins, the fibrogenic cytokine TGF-β1, as well as intermediate filaments. Cytokine receptor transcript levels were either not significantly changed or were found to be upregulated in LTCs (Fig. 8). RNA expression of the cytokines IL-1α, IL-1β, and IL-6 was higher in PSCs, whereas the mRNA encoding PDGF chains A and B were downregulated in activated primary cells. Interestingly, PPARγ transcript levels were low in PSCs, whereas LTCs expressed a marked amount of this nuclear receptor encoding RNA. Similar results were found in microarray experiments comparing the expression patterns of LTC-7 and PSC-7 (data not shown).

Expression results concerning genes of interest obtained from the array technique were confirmed by RT-PCR. RNA extracted from cultivated PSCs at different time points during the transformation process was compared with the

![Fig. 8. Gene expression profile comparing RNA levels in PSCs 14 days after isolation with LTC-14 using the microarray analysis. A: scatterplot of the average difference (Avg Diff) vs. fold change of RNA expression with LTC-14 as baseline is shown. Extracted total RNA of the respective cells was transcribed into first-strand cDNA. After second-strand synthesis, in vitro transcription was performed to obtain biotin-labeled cRNA samples. Chip hybridization was carried out according to the supplier’s instructions followed by staining with R-phycerythrin streptavidin. Fluorescence intensity was scanned, and results were scaled on the basis of total intensity with the Affymetrix chip software. The scatterplot shows valid gene expression data of a chip hybridization experiment with the average difference on a logarithmic scale and the fold change data on a standard scale. B: fold change values of transcript levels of functionally relevant gene groups expressed in PSCs cultured for 14 days vs. LTC-14 at baseline is shown. ECM, extracellular matrix.](http://ajpgi.physiology.org/)}
transcript levels in LTCs. Figure 9 shows selected images demonstrating RT-PCR products representing TGF-β1, IL-6, collagen type I, and PPARγ encoding RNA. The mRNA coding for collagen type I increased in parallel to the PSC activation, whereas TGF-β1 expression did not change significantly during PSC cultivation. In agreement with the microarray results, the transcript levels of collagen type I and TGF-β1 detected by RT-PCR were similar in activated PSCs and both LTC lines. IL-6 was expressed in PSCs at the same level, independent of the activation stage but down-regulated in LTCs.

PPARγ was highly expressed in early PSCs and downregulated during the transformation process, resulting in lower transcript levels in immortalized PSCs. In contrast, the SV40 large T antigen gene transfer obviously led to an upregulation of PPARγ in immortalized stellate cells (Fig. 9).

Fat storing. A characteristic feature of freshly isolated stellate cells is the storage of fat, which could be visualized by Oil Red O staining as shown in Fig. 10A. The transformation process induced by in vitro cultivation of primary cells was associated with a decrease of fat content resulting after 4 wk in the typical myofibroblastic appearance with only a few minute lipid droplets (Fig. 10B). The immortalized cell lines LTC-7 and LTC-14 were found to retain the fat storing capacity (Fig. 10, E and G).

Furthermore, Fig. 10 summarizes the results of Oil Red O-stained (14 days) cultured primary PSCs (C), LTC-7 (E), and LTC-14 (G) as well as their respective counterparts treated for 72 h with the PPARγ ligand PGJ2 (D, F, H) showing that the stimulation of PPARγ caused an increase of number and size of fat droplets in primary PSCs as well as in immortalized cell lines.

DISCUSSION

PSCs are known to undergo a myofibroblastic transformation within several days during in vitro cultivation associated with a functional activation (2, 4, 13, 14). It is accepted that after organ injury, similar processes occur leading to SMA positive PSCs, accompanied by an increased production of ECM (3, 14, 22, 25, 31, 32). Therefore, the understanding of the molecular mechanisms of this stellate cell-specific transition should provide the basis for the development of strategies to diminish SC activity, which might enable inhibition of organ fibrosis. Models are necessary to study the complex processes involved in the regulation of PSC transformation.
The purpose of our study was the establishment of immortalized rat pancreatic stellate cell lines with retained and stable characteristic features of primary cells. The most common approach for cell immortalization is the transduction of the SV40 large T antigen encoding gene (15). The molecular mechanisms of the LT-induced immortalization are complex. The respective outcome and the special features of immortalized cells appear to be random, likely influenced among other causes by the integration site of the LT gene and the transduced cell population (7, 28).

The morphology of native as well as activated PSCs demonstrated the characteristic features of primary stellate cells as previously described (2, 4). With the use of the retroviral LT gene transfer, PSCs could be successfully immortalized, resulting in two cell lines retaining their characteristics even after long-term passage in culture. PSCs were regularly positive for vimentin, independent of their activation status, showing the mesenchymal origin of the cells. Both LTC lines retained the high synthesis level of this intermediate filament and a similar intracellular organization.

Beyond the induction of SMA expression, PSC activation is accompanied by the formation of stress fibers. Regarding the morphology and the SMA expression, LTC-14 closely resembled the primary cells from which they were derived. In contrast, LTC-7 produced only a small amount of SMA without the typical fiber organization, and thus this cell line might reflect a transition phenotype characteristic for the early transformation status of quiescent to activated stellate cells. The differences between both cell lines in morphology and SMA expression remained constant throughout all passages so far.

The reaction of stellate cells to cytokines is an important feature for mediating fibrosis (3, 12, 20, 22, 25). Primary stellate cells are known to respond to PDGF by enhanced proliferation and by the induction of ECM synthesis in response to the profibrogenic cytokine TGF-β1 (3, 20, 22, 32). The treatment of LTCs with PDGF resulted in a concentration-dependent increase of proliferation, and TGF-β1 caused an elevated collagen production, indicating the responsiveness of immortalized cells to these cytokines.

The involvement of the nuclear hormone receptor PPARγ in the transformation process of HSCs is well documented (11). Originally, PPARγ was shown to be crucially involved in the glucose and lipid metabolism (29). Furthermore, the activation of PPARγ can promote differentiation of fibroblasts into adipocytes (17, 30). Recently, Masamune et al. (24) reported that ligands of PPARγ blocked the activation of PSCs as determined by decreased proliferation as well as diminished expression of SMA and collagen. Here, we have shown that the activation of PSCs, as described for HSCs, was associated with a decrease of PPARγ transcript levels, whereas receptor expression was upregulated in both LTC lines. This finding might explain the tendency toward a more sensitive response of LTCs to the PPARγ ligand PGJ2 affecting collagen synthesis. The transformation of stellate cells is accompanied by a loss of fat-containing vacuoles (4). We have shown a drastic reduction of Oil Red O-stained droplets during in vitro cultivation of primary cells, and after 4 wk, PSCs only occasionally contained lipid droplets. In contrast, the immortalized cell lines contained a similar amount of fat droplets as the early primary cells.

PSCs, as well as LTCs, responded to the stimulation of PPARγ with the incorporation of lipids resulting in an increase of number and size of fat droplets. Thus, in addition to the inhibitory effect of PPARγ stimulation on collagen production by PSCs, we found a characteristic feature of early stellate cells induced by a PPARγ ligand consisting in the increase of the fat content. In summary, the generated LTCs may be useful for investigating the molecular mechanisms of effects mediated by the nuclear receptor PPARγ on stellate cells.

With the use of the microarray technique, RNA expression profiles in primary and immortalized cell lines were determined, and genes of interest were further analyzed by RT-PCR. Regarding transcript levels of genes encoding ECM components, proteins involved in the signal transduction pathways, integrins, and intermediate filaments, no significant differences were detected between LTC lines and primary cell counterparts. The transcript levels of TGF-β1, of its receptors as well as of SMAD proteins, which are involved in the intracellular signal cascade activated by TGF-β1 (9, 35), were similar in PSCs and LTCs.

Generally, PSCs are regarded as targets for cytokine effects that are implicated in the pathogenesis of chronic pancreatitis (3, 22, 27, 31, 33). However, the synthesis of various cytokines by stellate cells themselves could be decisively involved in the perpetuation of an inflammatory process (1, 34, 37). In addition to the known expressions of IL-1β, IL-6, IL-8, and TGF-β (1, 12, 19) by stellate cells, the mRNA analysis also revealed the expression of IL-7, IL-15, IL-18, and PDGF. In this regard, the immortalized stellate cells showed a similar expression pattern. Thus the generated cell lines represent a perfect model system, because the basic features of primary PSCs are well retained.

In summary, we successfully established two rat pancreatic stellate cell lines that overcome many of the limitations in studying primary PSC cultures. LTC-14 corresponded morphologically and functionally to activated PSCs, whereas the LTC-7 line displayed morphological characteristics of an earlier stage of transformation.

These cell lines should facilitate further studies designed to characterize gene expression, protein synthesis, and intracellular signal transduction and to investigate effects of substances of interest on PSC functions. LTCs retaining the expression of essential proteins with respect to the fibrogenic function of stellate cells could be useful as an in vitro model for the development of therapeutic strategies.

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References


