Growth and Cell Cycle Abnormalities of Fibroblasts From Tangier Disease Patients

Wolfgang Drobnik, Gerhard Liebisch, Carola Biederer, Barbara Trümbach, Gerhard Rogler, Peter Müller, Gerd Schmitz

Abstract—We have investigated the abnormal proliferation and morphology of fibroblasts from patients with Tangier disease (TD), a high density lipoprotein (HDL) deficiency syndrome that is characterized by impairment of HDL-mediated lipid efflux and G protein–mediated signaling via phosphatidylinositol-specific phospholipase C (PI-PLC) and phospholipase D (PLD). TD fibroblasts displayed a 30% to 50% reduced in vitro growth rate and a 1.6-fold increased cell surface area. The response to different mitogens was diminished, and asynchronously growing TD fibroblasts showed 4.4 ± 0.3% S-phase and 19.1 ± 0.5% G2/M-phase cells compared with 9.7 ± 0.6% and 7.8 ± 0.5%, respectively, in controls. Monensin, but not brefeldin A, induced an S- and G2/M-phase distribution in control cells similar to that found in TD fibroblasts. This effect of monensin was accompanied by an increase of ceramide levels in controls, whereas TD fibroblasts already had a 2.5-fold increased basal ceramide concentration. Incubation of control cells with C2 ceramide and threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) mimicked the effect of monensin on the cell cycle. The inhibition of neither G protein function by pertussis toxin nor PLD by butanol resulted in a G2/M-phase arrest. Propranolol, known to increase phosphatidic acid levels, was ineffective in reversing the G2/M-phase arrest in TD fibroblasts. In addition, cDNA sequences and mRNA expression of the participants of PI-PLC or PLD signaling, ie, G-protein subunits α1, α2, and α3; phosphatidylinositol transfer proteins-α and -β; and ADP ribosylation factors 1 and 3 were found to be normal. Thus, growth and cell cycle abnormalities in TD fibroblasts are likely to be related to impaired Golgi function and sphingolipid signaling rather than inoperative G-protein signal transduction. Because PDMP was also found to decrease HDL3-mediated lipid efflux in control but not TD fibroblasts, similar pathways seem to be involved in the disturbances of lipid transport and growth retardation. (Arterioscler Thromb Vasc Biol. 1999;19:28-38.)

Key Words: Tangier disease ■ ceramide ■ Golgi apparatus ■ cell cycle ■ cholesterol efflux

Tangier disease (TD) is a rare, autosomal recessive disorder of cellular lipid and lipoprotein metabolism. Concomitant with a severe reduction of HDL levels in plasma, there is cholesteryl ester deposition in various tissues. Metabolic studies in TD patients have shown that the reduced levels of HDL are most likely due to an increased catabolism of HDL or its precursors. The underlying genetic defect affects intracellular lipid transfer processes, as indicated by the following observations: (1) In contrast to normal mononuclear phagocytes (MNPs), TD MNPs degrade internalized HDL completely in lysosomes. (2) On cholesterol loading with acetylated LDL, TD MNPs accumulate 2 types of unusual lysosome. (3) HDL3-mediated efflux of newly synthesized cholesterol and phospholipids is markedly reduced in TD fibroblasts. This defect in intracellular lipid transport can be normalized by 1,2-dioctanoylglycerol, a short-chain analogue of the second messenger 1,2-diacylglycerol, and by propranolol, a drug known to increase intracellular phosphatidic acid levels. Thus, the previously demonstrated impaired HDL3-mediated signal transduction in TD fibroblasts (ie, reduced activation of phosphatidylinositol-specific phospholipase C [PI-PLC] and a phosphatidylcholine-specific phospholipase D [PLD]) seems to be causally linked to defective HDL3-dependent lipid transport. Because both PI-PLC and PLD were found to be coupled to a pertussis toxin (PTX)–sensitive G protein, it is possible that the genetic defect in TD is associated with a member of this G-protein family.

However, a characteristic finding in different cell types of TD patients is an abnormal morphology of the Golgi apparatus. In MNPs and fibroblasts of TD patients, the cisternae of the trans-Golgi region are markedly dilated and the more dense form of the unusual lysosomes seems to degrade Golgi material. Thus, a dysfunction of the Golgi apparatus is very likely involved in the disturbed intracellular transport process. Moreover, recent progress in the understanding of Golgi function clearly indicates a close relationship between vesicular Golgi transport and signal transduction mechanisms.
The mammalian phosphatidylinositol transfer protein (PTTP) isoforms-α and -β have been shown to be required for the formation of secretory vesicles from the trans-Golgi network. The underlying mechanism probably involves the promotion of phosphatidylinositol-4,5-biphosphate synthesis, which seems to be critical for vesicular budding and is also essential for PI-PLC– and PLD-mediated signal transduction. In addition, ADP ribosylation factor (ARF), a small, monomeric G protein that is needed for the recruitment of coat proteins to the Golgi, has been shown to be an activator of PLD, and the need for ARF in vesicle budding can be bypassed by providing PLD. Thus, a disturbance in the regulation of phospholipid metabolism may link impaired Golgi function and signal transduction in TD. This hypothesis is supported by enhanced turnover of phospholipids in TD cells that results in a net increase of the cellular concentration of sphingomyelin and may also relate to the impaired signal transduction and Golgi traffic in TD cells. Sphingolipid-derived molecules are important second messengers with cross-talk to other signaling pathways, including signal transduction and Golgi traffic in TD cells.

Cell Culture and Growth Kinetics

Fibroblasts were routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1-glutamine, nonessential amino acids, and 10% FCS under 5% CO2 in a humidified incubator at 37°C. The experiments described herein utilized cells at passages 8 to 15. Contamination of cells with Mycoplasma was routinely tested for, and only Mycoplasma-free cells were used for experiments. For analysis of growth kinetics, cells were seeded into 6-well plates at a density of 10,000 cells per well and incubated with DMEM supplemented with 10% FCS or 0.5% BSA (negative control) for the times indicated in the figure legends. Cell numbers were determined by enzymatic dissociation with 0.04% trypsin in PBS and counted in a Neubauer chamber. Cell numbers on day 0 were determined 12 hours after seeding of 10⁵ cells per well. Viability of cells was determined by trypan blue exclusion.

Morphometric Analysis of Cultured Fibroblasts

Fibroblasts were grown to confluence on Laboratory-Tek chamber slides. They were fixed by immersion in 70% ethanol at 20°C and stained by incubation with a monoclonal antibody against α-tubulin and a fluorescence-labeled anti-mouse IgG. This procedure permitted very accurate identification of cell borders. The cell surface was measured by integration of the maximal surface area in a confocal laser scan microscope CSLM (Leitz).

Scanning Electron Microscopy

Control and TD fibroblasts were fixed in phosphate-buffered 3.5% glutaraldehyde solution, pH 7.4. Subsequent to fixation, the cells were rinsed 3 times with PBS; sequentially dehydrated in 30%, 50%, and 70% alcohol and 3 times in 99% alcohol; and dried by the critical point drying method. Immediately after critical-point drying, the cells were sputter-coated with a 20-A gold layer and visualized with a scanning electron microscope (Zeiss, Oberkochen).

PDGF and EGF Binding Assays

Fibroblasts (10⁵ cells) were seeded on 10-cm dishes and incubated for 24 hours with 10% FCS. Cells were then carefully rinsed 3 times with PBS and incubated for 2 hours with serum-free medium. Cells were subsequently incubated at 4°C with 0.2 to 10 nmol/L of [125I]-EGF or with 0.02 to 1.0 nmol/L of [125I]-PDGF-AB in HEPES buffer supplemented with 1% BSA. Nonspecific binding was determined by inclusion of a 200-fold excess of unlabeled human recombinant EGF or PDGF-AB. Thereafter, cells were washed extensively with buffer and lysed with 0.2% SDS. Aliquots were taken for gamma counting in a Pharmacia LKB gamma counter and for protein determination. Scatchard plots were constructed by using GraphPad software.

Isolation of Peripheral, Human MNCs

Peripheral, human MNCs from a healthy volunteer and a TD patient (patient 1) were isolated by leukapheresis. Subsequent to density gradient centrifugation over Ficoll/Hypaque, monocytes were separated by counterflow centrifugal elutriation. Elutriated monocytes that were >90% pure, as determined by immunophenotyping in an FACScan (Becton-Dickinson) analysis, were used for further analysis.

Cell Cycle Analysis

Determination of cell cycle distribution was performed by a modification of the method according to Vindelov et al. After treatment of cells (indicated in the text or the figure legends), they were washed twice with 2 mL PBS, detached with 0.04% trypsin in PBS, transferred to a Falcon tube, and centrifuged at 250g for 10 minutes. The resulting cell pellet was gently resuspended in 100 μL of a solution containing trypsin in a spermine tetrahydrochloride detergent buffer for the enzymatic digestion of cell membranes and cytoskeletons (solution A; Cycle Test PLUS DNA reagent kit).
incubated for 30 minutes at 37°C. Subsequently, 100 μL of a citrate buffer with spermine tetrahydrochloride containing a trypan inhibi-
itor and ribonuclease A was added (solution B; Cycle Test PLUS DNA reagent kit), and the mixture was incubated for 15 minutes at
room temperature to inhibit trypan activity and digest the DNA.
Finally, 100 μL of propidium iodide solution was added (solution C; Cycle Test PLUS DNA reagent kit) and incubated for at least 15
minutes on ice. The propidium iodide stoichiometrically binds to
DNA at a final concentration of ~125 μg/mL. The isolated and stained nuclei were directly analyzed and kept at 4°C in the dark.
Propidium iodide fluorescence was stable for ~4 hours under these
conditions. Flow-cytometric cell-cycle analysis was performed on an
FACScan (Becton-Dickinson) equipped with a doublet discrimina-
tion module and 15-mW argon laser using an excitation wavelength
at 488 nm and emission at 580 nm. The flow cytometer was
calibrated with ethanol-fixed chicken erythrocyte nuclei included in
the DNA quality control particle kit (Becton-Dickinson) according to
the instructions of the manufacturer. The cell cycle distribution (G1,
phase, S phase, and G2/M phase) of isolated nuclei was determined by
eclift software (Becton-Dickinson). Histogram analysis was performed using the R-Fit model.12 The coefficient of variation of the
G1 phase peak was <8%. Cell doublets were excluded by gating using the doublet discrimination module.

**Quantification of Ceramide Levels**

For the analysis of basal ceramide concentration, fibroblasts were grown to confluence. To investigate the influence of different drugs
on intracellular ceramide levels, confluent fibroblasts were treated
with the indicated agonist for 24 hours. Cells were then sonicated in
ice-cold PBS, and an aliquot was set aside for protein determination.
Subsequently, the lipids were extracted according to the method of Bligh and Dyer.30 Cellular lipids were separated by HPTLC. Samples
were dissolved in chloroform/methanol and applied, together with
an internal standard. For staining, HPTLC plates were dipped in 10%
H3PO4 for 12 seconds and heated to 170°C for 15 minutes on ice. The propidium iodide stoichiometrically binds to
DNA at a final concentration of ~125 μg/mL. The isolated and stained nuclei were directly analyzed and kept at 4°C in the dark.
Propidium iodide fluorescence was stable for ~4 hours under these
conditions. Flow-cytometric cell-cycle analysis was performed on an
FACScan (Becton-Dickinson) equipped with a doublet discrimina-
tion module and 15-mW argon laser using an excitation wavelength
at 488 nm and emission at 580 nm. The flow cytometer was
calibrated with ethanol-fixed chicken erythrocyte nuclei included in
the DNA quality control particle kit (Becton-Dickinson) according to
the instructions of the manufacturer. The cell cycle distribution (G1,
phase, S phase, and G2/M phase) of isolated nuclei was determined by
eclift software (Becton-Dickinson). Histogram analysis was performed using the R-Fit model.12 The coefficient of variation of the
G1 phase peak was <8%. Cell doublets were excluded by gating using the doublet discrimination module.

**Determination of Lipid Efflux From Fibroblasts**

**Metabolic Labeling of Fibroblasts**

On reaching confluence, fibroblasts were rinsed and incubated for 48 hours in DMEM containing 10% FCS supplemented with 3 μCi/mL
[3H]choline chloride to label choline-containing phospholipids and
1.5 μCi/mL [3H]cholesterol. For the last 12 hours of pulse incubation,
40 μmol/L PDMP was supplemented to the medium when indicated.
After incubation with the radioactive tracer, the medium was
removed, and cells were rinsed twice with PBS supplemented with
0.5% BSA and once with PBS alone.

**Determination of Lipoxygenase Activity**

Cells were incubated for 12 hours at 37°C in DMEM containing 0.5% BSA supplemented with 100 μg/mL HDL, with or without
40 μmol/L PDMP. After incubation, the media were removed and
centrifuged at 800g to precipitate any detached cells. Cells were
rinsed 3 times with PBS and lysed with 0.2% SDS. Aliquots of cell
lysates were taken for protein determination. Lipids were extracted from supernatant media and cell lysates according to the method of Bligh and Dyer.30 H and 14C radioactivities were measured by liquid
scintillation counting in total lipid extracts from cell lysates and in
the incubation media for distinguishing 14C-labeled cholesterol from
1H-labeled phospholipids. Lipid efflux in response to HDL is expressed
as the percentage of "total radioactivity" that appeared in the medium
after the chase period: [disintegrations per minute (dpm) in medium/
(dpm in medium+ dpm in cells)]. Specific HDL-mediated efflux was
calculated as the difference between the efflux to "HDL +0.5% BSA"
minus the nonspecific efflux to "0.5% BSA alone."

**RNA Isolation and RT-PCR Conditions**

Fibroblasts between passages 8 and 15 were grown to confluence, and total RNA was isolated by using previously described procedures. G1, G2, G3, ARF1, ARF3, PITP-a, and PITP-b–specific DNA probes were
generated using corresponding primer pairs in a PCR reaction (Expand
high-fidelity PCR system). cDNA was generated using a commercially
available RT kit (Promega). The primers were derived from the 5’ and
3’-untranslated regions where sequences are less conserved. The following
primer pairs were used for amplification: for G1, Ga1-3, 5’-AACATCT-
TTGTCAGTTCCAGAT-3’; Ga1-5, 5’-CTAGGAGAGAAAGG-A
TTCCCCT-3’; for G2, Ga2-3, 5’-CATACCGGAGGGCCCGCT-
GCC-3’; Ga2-5, 5’-GGCCGCGGGCGGACCGGATA-3’; for Ga3,
Ga3-3, 5’-GATACTTACATACGCTTACA-3’; Ga3-5, 5’-GAGTTCG-
GGCGCGCTTCCCTTC-3’; for ARF1, Ga1-3, 5’-TTGGATCCGTG-
TCCTCCATGCACA-3’; ARF1-5, 5’-GTTGACCTGCAGAAGAGA-
GAACAG-3’; for ARF3, ARF3-3, 5’-TTGGATCCAGGCAGCTG-
CTGTGACGAT-3’; ARF3-5, 5’-CCGAAATTCCTGGCCACACTTGC-
GAGAAG-3’; for PITP-a, PITP-a-3, 5’-TTGGTACCTGCTATGACGAC-
AATGGGATG-3’; PITP-a-5, 5’-CCGAAATTCATGGACACAGA-
ACGCTGCGTCGG-3’; for PITP-b, PITP-b-3, 5’-GTTGATCTAC-
TGGTGAAGAGATG-3’; PITP-b-5, 5’-CTGAAATTCTCGG-
CAAAGCTCAGATGAC-3’.

The cycling conditions were as follows: 2 minutes at 94°C
followed by 10 cycles of 10 seconds at 93°C, 30 seconds at 62.5°C,
60°C, and 61.0°C for Ga1, Ga2, and Ga3–specific primers, respec-
tively, and 30 seconds at 59.0°C, 60.0°C, and 61.0°C for ARF1, ARF3,
PITP-a, and PITP-b–specific primers; 55 seconds at 72°C; and an additional 30 cycles with an incremental increase of the
elongation time by 20 seconds in each cycle. After a final extension
for 10 minutes at 72°C, the PCR products were analyzed on agarose gels. The fragments of interest were gel-purified (QIAEXII gel
extraction kit), cloned into plUC18 plasmids, and sequenced using an
automated fluorescence-based sequencer.

**Northern Blot Analysis**

For northern blot analysis, 15 μg (G1 to 3) and 10 μg (PITP-a/β and ARF1/3) of total RNA were separated on 1.2% formaldehyde
agarose gels, and RNA was transferred to a nylon membrane and
immobilized by UV cross-linking.31 Polabeled, random-primed DNA probes were prepared using the cloned cDNA fragments for G1, G2, G3, ARF1, ARF3, and ARF3. Northern blots were hybrid-
ized overnight at 65°C (G proteins) or 60°C (PITPs and ARFs) in
50 mmol/L PIPES (pH 6.5), 100 mmol/L NaCl, 50 mmol/L NaPO4
(pH 7.0), 1 mmol/L EDTA, and 5% SDS in the presence of the probe.
Blots were washed in 5% SDS and 1× SSC for 10 minutes at
room temperature and subsequently in 5% SDS and 1× SSC for 20
minutes at 65°C (G proteins) or 60°C (PITPs and ARFs). The blots were
exposed to x-ray film at −80°C for 6 to 47 hours with intensifier screens.

**Protein Determination**

Protein quantification was performed according to the method of
Smith et al.32

**Statistical Analysis**

Results are expressed as mean±SEM. The overall mean was
calculated from the mean values of multiple determinations per-
formed on the indicated number of TD patients and control subjects.
Statistical significance was assessed by Student’s t test for paired or
unpaired values and set at P<0.05.

**Results**

**Morphological Characterization of TD Fibroblasts**

Because previous studies had indicated that fibroblasts from TD patients appeared to be larger than those of control subjects, the cells’ morphologies were investigated by mor-
phometry. At least 2 days after cells had reached confluence, the average surface area of TD fibroblasts (5015 ± 215 μm², n = 2) was significantly greater (P < 0.01) than that of control fibroblasts (3090 ± 110 μm², n = 2).

Figure 1 shows a representative scanning electron microscopy picture of fibroblasts from control subjects and TD patients. Control fibroblasts were homogeneous in size and orientation (Figure 1A). In contrast, TD fibroblasts grew heterogeneously in culture. They were more variable in size and showed irregular orientation on the culture dish (Figure 1B). These differences were independent of the passage number of the cells.

### Analysis of In Vitro Growth of Fibroblasts From TD Patients in Response to FCS

On the basis of the abnormal morphology and the empirical observation of a reduced in vitro growth of TD fibroblasts, we performed detailed analysis of cell proliferation. Growth kinetics of fibroblasts of 4 different control subjects and 3 TD patients cultured in DMEM supplemented with 10% FCS were determined over a period of 10 days (Figure 2). All cells used in the experiments had comparable passage numbers. As of day 3 in culture, the cell numbers of control fibroblasts (25,981 ± 3162 cells per well) were significantly (P < 0.002) higher than those of TD fibroblasts (14,729 ± 1879 cells per well; Figure 2). After 8 days in culture the number of control fibroblasts per well reached a maximum of 103,026 ± 25,429, whereas the average cell number of TD fibroblasts was only 29,967 ± 5948 (Figure 2). In culture medium supplemented with 0.5% BSA instead of 10% FCS, neither control nor TD fibroblasts showed any significant variations in cell number over the entire 8-day culture (Figure 2). The cell numbers on day 0, determined 12 hours after seeding of 10,000 cells per well, were 8870 ± 195 for TD fibroblasts and 9475 ± 180 for control fibroblasts.

The growth retardation of TD fibroblasts could not be overcome by the addition of conditioned medium derived from cultures of control fibroblasts. TD fibroblasts were incubated with conditioned medium either with or without the addition of fresh FCS for 7 days, and under both conditions the numbers of TD fibroblasts were ≈50% of the control cell counts (data not shown).

### Mitogenic Effects of EGF and PDGF on TD Fibroblasts

The mitogenic effects of 2 growth factors were tested by cell cycle analysis. As shown in Figure 3, the incubation of asynchronously growing control fibroblasts with 30 ng/mL EGF or PDGF-AB for 24 hours further increased the proliferation of these cells. This effect is indicated by a marked increase in the percentage of cells in the S and G2/M phases (Figure 3). In contrast, TD fibroblasts did not respond to
either EGF or PDGF-AB with an increase of cells in the S or G2/M phase (Figure 3).

**Binding of EGF and PDGF to TD Fibroblasts**
To investigate whether a reduced expression of growth factor receptors might be responsible for the impaired mitogenic response of TD fibroblasts to EGF and PDGF-AB, the binding of these mitogens to their cellular receptors was determined. No significant differences were found between the cell lines for Bmax and Kd. In a representative experiment, Bmax values for PDGF-AB and EGF were 8.13 and 1.15 pmol/mg cell protein in control fibroblasts and 8.01 and 1.35 pmol/mg cell protein in TD fibroblasts, respectively.

**Cell Cycle Distribution in TD Fibroblasts**
Because the cell cycle studies with PDGF-AB and EGF indicated a substantially increased number of G2/M-phase cells in TD cultures, the cell cycle distribution of synchronized as well as asynchronously growing fibroblasts of different passages and from an extended number of TD patients (n=3) and control subjects (n=4) was determined in multiple experiments (Figure 4). The results confirmed the findings shown in Figure 3. Compared with control fibroblasts, TD fibroblasts showed a significantly higher number of total cells in the G2/M phase. This result was independent of whether the cells were synchronously (6.6±0.4% versus 19.5±2.8%, P<0.003; Figure 4A) or asynchronously

---

**Figure 3.** Effects of EGF and PDGF-AB on cell cycle distribution in asynchronously growing control and TD fibroblasts. Control (open bars) and TD (filled bars) fibroblasts were grown for 72 hours in DMEM and 10% FCS and then incubated for a further 24 hours in the same medium with the indicated concentration of EGF (A, B) or PDGF-AB (C, D), respectively. Data for cells in the S phase (A, C) and G2/M phase (B, D) are expressed as percent of total cells. Each bar represents mean±SEM of 2 experiments performed in duplicate on fibroblasts from 2 control subjects and 2 TD patients, respectively.
(7.8±0.5% versus 19.1±0.5%, P<0.001; Figure 4B) growing. In addition, the percentage of asynchronously growing cells in the S phase was significantly reduced in TD fibroblasts (9.7±0.6% versus 4.4±0.3%, P<0.003; Figure 4B). After 24-hour incubation of asynchronously growing control fibroblasts with hydroxyurea (2.5 μmol/L), a drug known to halt the cell cycle in the S phase, nearly all cells disappeared from the G2/M phase (0.9±0.7%) and accumulated in the G1 (86.6±1%) or S (12.5±1.7%) phase of the cell cycle (n=2). In contrast, the same treatment of TD fibroblasts reduced the percentage of cells in the G2/M phase only gradually (17.6±1.5% versus 21.8±2.7%; n=2). Prolonged incubation with hydroxyurea for up to 5 days did not result in a further decline of G2/M-phase cells in TD fibroblasts.

Influence of Monensin and BFA on Intracellular Ceramide Concentration

To evaluate the effect of monensin on intracellular ceramide concentrations, asynchronously growing (72 hours, 10% FCS) control and TD fibroblasts were incubated with different concentrations of monensin for 24 hours. In control fibroblasts monensin significantly (P<0.05, n=2) increased ceramide levels by 35% at 20, 42% at 40, and 60 μmol/L, respectively. The already 2.5-fold increased basal ceramide concentrations in TD fibroblasts were not significantly affected. Incubation with 10 μmol/L BFA had no effect on intracellular ceramide concentrations in control cells (n=2).

Influence of Modulators of Intracellular Ceramide Concentration on Cell Cycle Distribution

The relationship between the increased ceramide concentrations and the cell cycle abnormalities in TD fibroblasts was investigated by analyzing the influence of PDMP, an inhibitor of glucosylceramide synthase,25 and of C2 ceramide on cell cycle distribution. PDMP, which elevates intracellular ceramide concentrations, significantly decreased the percentage of S-phase cells in control fibroblasts at 40 μmol/L (Figure 6A) and increased G2/M-phase cells from 7.0±0.8% without PDMP to 11.9±0.9% at 10 μmol/L and to 8.1±0.7% at 40 μmol/L (Figure 6B). In contrast to control fibroblasts, cell cycle distribution in TD fibroblasts was not significantly affected (Figure 6A and 6B). To exclude the possibility that these effects of PDMP were mediated by depletion of higher glycosphingolipids and not due to increased ceramide concentrations, a short-chain analogue of natural ceramide, C2 ceramide, was tested for its influence on the cell cycle. C2 ceramide at 40 μmol/L significantly decreased the number of S-phase cells in control fibroblasts (Figure 6C) and increased the percentage of cells in the G2/M phase at 10 as well as at

Influence of Golgi Function on Cell Cycle Distribution

To investigate an involvement of Golgi function in the growth and cell cycle abnormalities, we analyzed the influence of monensin and BFA on cell cycle distribution. In control fibroblasts, monensin but not BFA significantly decreased the percentage of S-phase cells, reaching 11.8±0.9% at 100 μmol/L monensin compared with 16.6±0.6% without monensin (Figure 5A). In TD fibroblasts, however, the already low percentage of cells in the S phase was not affected (Figure 5A). Concomitantly, in control fibroblasts monensin markedly increased the percentage of cells in the G2/M phase in a concentration-dependent manner (Figure 5B). The percentage of cells in the G2/M phase increased from a basal level of 8.4±0.2% without monensin to 23.9±1.2% with 100 μmol/L monensin, which was very similar to those values found in TD fibroblasts under basal and monensin-containing conditions (Figure 5B). Furthermore, similar to TD fibroblasts, the number of G2/M-phase cells in monensin-treated control fibroblasts could not be decreased by treatment with hydroxyurea (2.5 μmol/L) for 24 hours. In contrast to monensin, BFA at the tested concentrations did not significantly affect the cell cycle distribution in either control or TD fibroblasts (Figure 5C and 5D).

Determination of Basal Ceramide Concentration in TD Fibroblasts

Because TD cells are known to have disturbed sphingolipid metabolism and ceramide is known to inhibit cell proliferation as well as Golgi function, the intracellular concentration of ceramide was determined in confluent cultures. Ceramide levels were found to be significantly increased in TD compared with control fibroblasts, with 4.5±0.7 compared with 1.8±0.2 nmol/mg cell protein, respectively (P<0.005 for control versus TD). These results represent mean±SEM values from 3 TD patients and 5 control subjects, respectively.

**Figure 4.** Cell cycle distribution of synchronously or asynchronously growing control and TD fibroblasts. Control (open bars) and TD (filled bars) fibroblasts were either synchronized (A) for 48 hours in DMEM and 0.5% BSA or incubated with 10% FCS in DMEM for 72 hours (B, asynchronously growing cells), and then cell cycle distribution was determined. Data for cells in G1, S, and G2/M phases are expressed as percent of total cells. Each bar represents mean±SEM of 4 and 3 experiments, each performed multiple times, from fibroblasts of 4 control subjects and 3 TD patients, respectively. ***P<0.003 for control versus TD.
40 μmol/L (Figure 6D). Like PDMP, C2 ceramide did not affect cell cycle distribution in TD fibroblasts (Figure 6C and 6D). The influence of PDMP and C2 ceramide on the number of control fibroblasts in the G2/M phase was confirmed by experiments in which control cells were synchronized with 0.5% BSA in the presence of either substance. Similar to the effect on asynchronously growing fibroblasts, the percentage of G2/M-phase cells was significantly increased in synchronized cells after 48-hour treatment (data not shown).

**Influence of PTX and Modulators of the PLD Signaling Pathway on Cell Cycle Distribution and Intracellular Ceramide Concentration**

To determine a potential role of impaired Gα-protein–mediated signal transduction and PLD signaling, the influence of PTX, an inhibitor of Gα proteins; butanol, a competitive substrate of PLD that reduces PLD-catalyzed production of phosphatidic acid; and propranolol, an inhibitor of phosphatidic acid hydrolase, on cell cycle distribution was investigated (the Table). Butanol at a concentration of 0.3% did not significantly affect cell cycle distribution of control and TD fibroblasts (the Table). Additional experiments showed that even 1.0% butanol did not induce a Gα/M-phase arrest (data not shown). Propranolol at 100 μmol/L and PTX at 100 ng/mL reduced the percentage of cells in the S phase in both control and TD fibroblasts, while the number of Gα/M-phase cells was unaffected (the Table). Furthermore, neither butanol nor PTX increased intracellular ceramide concentrations (data not shown).

**Influence of PDMP on HDL₃-Mediated Lipid Efflux**

Because TD fibroblasts are characterized by a marked impairment of HDL₃-mediated cholesterol and phospholipid efflux, the influence of PDMP (40 μmol/L) on these lipid

---

**Figure 5.** Effects of monensin and BFA on cell cycle distribution in asynchronously growing control and TD fibroblasts. Control (○) and TD (●) fibroblasts were grown for 72 hours in DMEM and 10% FCS and then incubated for a further 24 hours in the same medium with the indicated concentrations of monensin (A, B) or BFA (C, D), respectively. Data for cells in the S (A, C) and G2/M (B, D) phases are expressed as percent of total cells. Each data point represents mean ± SEM of 3 experiments, each performed in duplicate, on fibroblasts from 3 control subjects and 3 TD patients, respectively.
Transport processes was analyzed. As shown in Figure 7, PDMP significantly decreased specific HDL$_3$-mediated efflux of [14C]cholesterol and [3H]phospholipids in control cells, whereas the already reduced efflux in TD fibroblasts was not further diminished.

Cloning and Sequencing of G$_{ai1}$, G$_{ai2}$, and G$_{ai3}$ and Determination of the mRNA Expression Levels of These G-Protein Subunits in TD Fibroblasts

To determine whether mutations in G$_{ai1}$, G$_{ai2}$, or G$_{ai3}$ accounted for the impaired signal transduction or the cell cycle and growth abnormalities in TD cells, we cloned and sequenced the corresponding regions. cDNAs were reverse-transcribed from RNA isolated from cultured fibroblasts from 4 TD patients. DNA sequencing revealed no alterations at the cDNA level with an impact on the amino acid sequence. Furthermore, analysis of mRNA expression from fibroblasts of 4 healthy controls and 4 TD patients demonstrated similar levels and normal sizes for all 3 transcripts in TD fibroblasts when compared with controls.

Cloning, Sequencing, and Determination of mRNA Expression of ARF1 and 3 as Well As PITP-$\alpha$ and -$\beta$ in TD Fibroblasts

Because the monomeric G proteins of the ARF family and the PITPs are related to PLD and PI-PLC signaling as well as

Figure 6. Effects of PDMP and C2 ceramide on cell cycle distribution in asynchronously growing control and TD fibroblasts. Control (open bar) and TD (filled bar) fibroblasts were grown for 72 hours in DMEM and 10% FCS and then incubated for a further 24 hours in the same medium with the indicated concentrations of PDMP (A, B) or C2 ceramide (C, D), respectively. Data for cells in the S (A, C) and G$_2$/M (B, D) phases are expressed as percent of total cells. Each bar represents mean±SEM of 4 and 2 experiments, performed in duplicate, on fibroblasts from 4 control subjects and 2 TD patients, respectively. *P<0.05 for treated (10 or 40 \(\mu\)mol/L of PDMP or C2 ceramide) versus untreated cells (0 \(\mu\)mol/L).
substance for 24 hours in DMEM with 10% FCS, the cell cycle distribution was
with [14C]cholesterol and [3H]choline for 48 hours. The last 12
 CONTROL (open bar) and TD (filled bar) fibroblasts were labeled

<table>
<thead>
<tr>
<th></th>
<th>Control Fibroblasts</th>
<th>TD Fibroblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S Phase, %</td>
<td>G2/M Phase, %</td>
</tr>
<tr>
<td>Basal</td>
<td>10.0±0.1</td>
<td>5.0±1.2</td>
</tr>
<tr>
<td>0.3% Butanol</td>
<td>8.3±1.0</td>
<td>5.4±0.5</td>
</tr>
<tr>
<td>Basal</td>
<td>7.9±0.6</td>
<td>7.8±0.4</td>
</tr>
<tr>
<td>100 μmol/L propanolol</td>
<td>3.0±0.7*</td>
<td>8.2±0.4</td>
</tr>
<tr>
<td>Basal</td>
<td>13.7±2.9</td>
<td>7.9±0.7</td>
</tr>
<tr>
<td>100 ng/mL PTX</td>
<td>1.6±0.3*</td>
<td>5.8±1.1</td>
</tr>
</tbody>
</table>

Independent of cell passage number, a markedly higher percentage of TD fibroblasts were found in the G/M phase of the cell cycle, which was characteristic for fibroblasts from different TD patients, independent of whether the former were in a growing or resting state. The failure to synchronize TD fibroblasts with hydroxyurea, a drug known to block cells in the S phase of the cell cycle, indicates that the increased percentage of G2/M-phase cells in TD fibroblasts may be due to a real arrest rather than a delay in G/M. The G/M arrest may be linked to the higher average cell surface area in cultures of TD fibroblasts, because enlarged size is a typical feature of G2/M-phase cells.

An arrest in the G/M phase of the cell cycle has so far been described after treatment of cells with DNA-damaging drugs and is found in mammalian cells after irradiation or treatment with certain cytostatics. The present study indicates that monensin treatment of cultured fibroblasts of healthy controls can also induce an accumulation of cells in the G/M phase of the cell cycle. Monensin, an Na+ ionophore capable of causing collapse of Na+ and H+ gradients, has gained widespread acceptance as an inhibitor of Golgi function, with its most profound effects on the transcisternae of the Golgi apparatus stacks. The concentration-dependent ability of monensin to convert control fibroblasts to a TD phenotype (ie, increased G2/M-phase cells and reduced S-phase cells) suggests that an impaired Golgi function in TD fibroblasts may be associated with the cell cycle and growth abnormalities. This concept is supported by both the absent effect of monensin in TD fibroblasts as well as the previously demonstrated appearance of hyperplastic and dilated trans-Golgi elements in fibroblasts and MNPs of TD patients. Similarly, swelling of trans-Golgi cisternae is the most consistent visual in vitro demonstration of a monensin-induced effect on a membranous cell compartment. In contrast, inhibition of Golgi function by BFA, which is mainly characterized by the redistribution of Golgi components back to the endoplasmic reticulum, had no effect on the cell cycle. Other cellular processes that can be inhibited by monensin and that are known to involve the Golgi apparatus include endoplasmic reticulum, the processing of acetylated LDL, and the transport of newly synthesized sphingomyelin and glucosylceramide to the plasma membrane and are also impaired in cells of TD patients (impaired transport of sphingomyelin and glucosylceramide in TD fibroblasts are from D.M. et al, unpublished data, 1997). Recently, a reduced HDL-mediated cholesterol efflux, which is a characteristic finding in TD fibroblasts, was found after disruption of Golgi function in fibroblasts of healthy subjects. However, monensin also affects compartments of the endocytic pathway by alteration of the vesicular pH and although the lysosomotropic amine chloroquine showed no G2/M-arresting effect and the pH of TD cells was normal (W.D. et al, unpublished data,
Ceramide has been described as a negative regulator of the cell cycle,\textsuperscript{25, 26, 41} and was recently shown to inhibit Golgi transport processes and to induce dilatation of the trans-Golgi region similar to that seen in TD fibroblasts.\textsuperscript{24} Furthermore, ceramide is known to inhibit PLD, which has been suggested to critically participate in vesicular traffic.\textsuperscript{42} Thus, the 2-fold increased ceramide level in TD fibroblasts can potentially link both the impaired Golgi function and the cell cycle abnormalities in TD cells. In accordance with this hypothesis, the effect of monensin on cell cycle distribution in control cells was accompanied by an increase of ceramide concentration. However, independent of whether ceramide is involved in abnormal Golgi function of TD fibroblasts or not, our data argue for ceramide’s role in mediating the G\textsubscript{2}/M-phase arrest and the reduced growth in these cells as well as in the monensin-treated control cells. This conclusion is supported by the following observations: (1) PDMP, an inhibitor of glucosylceramide synthase,\textsuperscript{25} which is known to increase intracellular ceramide levels, induced a significant increase in G\textsubscript{2}/M-phase cells in fibroblasts from control subjects but not from TD patients. (2) This PDMP effect was reproduced by C2 ceramide, and it is thus unlikely that reduced synthesis of glycosphingolipids is responsible for the effect of PDMP. Recently, similar findings were reported in 3T3 fibroblasts, wherein a short-chain analogue of ceramide as well as PDMP was able to induce a G\textsubscript{2}/M-phase arrest.\textsuperscript{23} (3) Furthermore, our data support previous reports demonstrating ceramide as a negative regulator of cell growth,\textsuperscript{41} since ceramide and PDMP at 40 \textmu mol/L markedly reduced the number of growing control fibroblasts in the S phase to a level comparable to that observed in TD cells. However, it cannot be excluded that the observed effects of C2 ceramide and PDMP are mediated by some downstream metabolites of ceramide. Thus, the present data suggest that either the increased ceramide by itself or some downstream sphingolipid mediators in TD fibroblasts contribute to the altered cell cycle distribution and the reduced mitogenic response of these cells. Although a general growth retardation is not observed in TD patients, impairment of cell cycle regulation may nonetheless occur under certain conditions in vivo. Thus, one can speculate whether a reduced mitogenic response of certain cells may be related to the lack of excessively increased atherogenic risk despite the nearly total absence of HDL. A functional role for sphingolipids in the pathomechanism of altered cellular function in TD is further supported by the recent observation that HDL-mediated cholesterol efflux occurs via these membrane domains.\textsuperscript{31}

Owing to the small pedigrees, molecular biology approaches that were successfully used to identify the genetic defects of other diseases will not be promising in the currently available TD families. Therefore, we tried to reduce the number of potential candidate genes by elucidating the functional interrelation of the different cellular abnormalities. The presented data contribute to this approach by linking Golgi function and sphingolipid signaling to a characteristic growth and cell cycle abnormality as well as the reduced HDL\textsubscript{3}-mediated cholesterol efflux observed in TD fibroblasts.

### Acknowledgments

This work was supported by Deutsche Forschungsgemeinschaft (DFG DR 348/2-1) (to Wolfgang Drobnik). The expert technical assistance of Stella Potra is greatly appreciated. This study was possible only with the continuing cooperation of the patients.

### References


9. Walter M, Gerdes U, Seedorf U, Assmann G. The high density lipoprotein- and apolipoprotein A-I-induced mobilization of cellular cho-
Tangier Disease and the Cell Cycle

Lebester is impaired in fibroblasts from Tangier Disease subjects. Biochim Biophys Acta. 1994;205:850–856.


Growth and Cell Cycle Abnormalities of Fibroblasts From Tangier Disease Patients
Wolfgang Drobnik, Gerhard Liebisch, Carola Biederer, Barbara Trümbach, Gerhard Rogler,
Peter Müller and Gerd Schmitz

doi: 10.1161/01.ATV.19.1.28
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272
Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://atvb.ahajournals.org/content/19/1/28

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published
in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the
Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which
permission is being requested is located, click Request Permissions in the middle column of the Web page
under Services. Further information about this process is available in the Permissions and Rights Question and
Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online
at:
http://atvb.ahajournals.org//subscriptions/