Growth and Cell Cycle Abnormalities of Fibroblasts From Tangier Disease Patients

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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 HDL₃-mediated lipid efflux and Gprotein-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

T angier 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.¹ 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.²-³ 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.⁴⁻⁵ (2) On cholesterol loading with acetylated LDL, TD MNPs accumulate 2 types of unusual lysosome.⁶ (3) HDL₃-mediated efflux of newly synthesized cholesterol and phospholipids is markedly reduced in TD fibroblasts.⁷⁻⁹ 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.¹⁰ Thus, the previously demonstrated impaired HDL₃-mediated signal transduction in TD fibroblasts (ie, reduced activation of phosphatidylinositol-specific phospholipase C [PI-PLC])¹¹,¹² and a phosphatidylcholine-specific phospholipase D [PLD])¹³ 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.² 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.¹⁴
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. Furthermore, ceramide, the initial signal transduction and Golgi traffic in TD cells. Additionally, cDNA sequences and mRNA expression of G₁/S and G₂/M transitions of the cell cycle. 

Methods

Subjects

Cutaneous fibroblasts were obtained from 4 patients homozygous for TD. Patient 1, female, aged 64 (triglycerides, 2.94 to 4.89 mmol/L; cholesterol, 2.02 to 2.67 mmol/L); patient 2, male, aged 57, brother of patient 1 (triglycerides, 1.58 to 2.24 mmol/L; cholesterol, 1.16 to 1.50 mmol/L); patient 3, a 42-year-old Pakistani aged 57, brother of patient 1 (triglycerides, 1.58 to 2.24 mmol/L; cholesterol, 1.16 to 1.50 mmol/L); patient 2, male, aged 57, brother of patient 1 (triglycerides, 1.58 to 2.24 mmol/L; cholesterol, 1.16 to 1.50 mmol/L). Subjects who underwent abdominal surgery. Cells were used for experiments.

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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) and QIAEXII gel extraction kit was from Qiagen. X-OMAT x-ray film was obtained from Kodak. Hybond-N, [3H]-PDGF-AB, [3H]-EGF, [3P]ATP, [3H]choline, and [14C]cholesterol were from Amersham.
incubated for 30 minutes at 37°C. Subsequently, 100 μL of a citrate buffer with spermine tetrahydrochloride containing a trypsin inhibitor 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 trypsin activity and digest the RNA. 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 FACSScan (Becton-Dickinson) equipped with a double discrimination module and 15-nW argon laser using an excitation wavelength at 488 nm and emission at 580 nm. The flow cytometer was calibrated with ethanol-fixed human erythrocyte nuclei included in the DNA quality control particle kit (Becton-Dickinson) according to the instructions of the manufacturer. The cell cycle distribution (G1, G2, and S phase) was determined by using elfit software (Becton-Dickinson). Histogram analysis was performed using the R-Fit model.32 The coefficient of variation of the G1 phase peak was ≤8%. Cell doublets were excluded by gating using the double 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 and applied, together with external standards, to 10×20-cm silica gel HPTLC plates (Merck) by using a capillary dispenser (Camag). Separation of ceramide was performed twice with chloroform/methanol/acetic acid, 19:9:1, vol/vol/vol.31 A standard curve was prepared by using ceramide type III (the mean molecular weight was estimated according to the manufacturer’s specifications). Ceramide type IV was used as an internal standard. For staining, HPTLC plates were dipped in 10% vol/vol/vol.31

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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-α, and PITP-β–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′-AAACAATT-TGTTGAAATCCGGATGAT-3′; Ga1-5, 5′-CTAGGAGAGAAGGAATCC-ATTCCTC-3′; for G2, Ga2-3, 5′-CATACCCCGAGGCCCCGCT-GCCC-3′; Ga2-5, 5′-GGCACCAGGCGGACGGGATG-3′; for G3, Ga3-3, 5′-TGAAATTTCCACTAACCCTGCT-3′; Ga3-5, 5′-GAATCCGGCGCGCTGGCTTCCCTC-3′; for ARF1, ARF3-3, 5′-TTGGATCTCGTGTTCTCCATGTGCACA-3′; ARF1-5, 5′-GTGAAATCCGGCGGACGGAATGGAAG-3′; for ARF3-5, 5′-TGAGCTCCGCGCGCTGGCTTCTC-3′; for PITP-α, PITP-α-3, 5′-TTGGATCTCGATCGCTTGCATGCCCAGCACA-3′; for PITP-β, PITP-β-3, 5′-GGCAATCTGCAGGACGACAGGA-GCAG-3′; for PITP-α-5, 5′-GGCAATCTGCAGGACGACAGGACAGGA-GCAG-3′; for PITP-β, PITP-β-5, 5′-CTGAAATCTCCGTCAGAACATAAGTACGCTGTA-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, 62°C, and 61°C for G1, G2-, and G3-specific primers, respectively, and 30 seconds at 59°C, 62°C, and 61°C for ARF1-, ARF3-, and PITP-α- and PITP-β–specific primers; 55 seconds at 72°C; and an additional 30 seconds 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 pUC18 plasmids, and sequenced using an automated fluorescence-based sequencer.

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 performed 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-
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

Figure 1. Scanning electron photomicrographs of control (A) and TD (B) fibroblasts. Both photomicrographs were taken from dishes containing cultures at confluence (magnification ×260).

Figure 2. Line graphs showing growth kinetics of control (○, ●) and TD (■, □) fibroblasts in response to 10% FCS. Cells (10,000) were seeded in 6-well dishes and incubated with DMEM and 0.5% BSA with (○, ●) or without (■, □) 10% FCS. Cells were counted at days 2, 3, 6, 8, and 10. Results represent mean ± SEM of 4 and 3 experiments, each performed in triplicate, on fibroblasts from 4 normal subjects and 3 TD patients, respectively.

Figure 3. Graph showing the effect of EGF (30 ng/mL) or PDGF-AB (10 ng/mL) on the growth of control and TD fibroblasts. Cells were incubated with growth factors for 24 hours, and cell cycle analysis was performed.
either EGF or PDGF-AB with an increase of cells in the S or G2/M phase (Figure 3).

**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.

**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

**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 $B_{\text{max}}$ and $K_d$. In a representative experiment, $B_{\text{max}}$ 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.
more, similar to TD fibroblasts, the number of G2/M-phase and monensin-containing conditions (Figure 5B). Further-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.

**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±18%, 42±18%, and 45±17% at 20, 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
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 G protein–mediated signal transduction and PLD signaling, the influence of PTX, an inhibitor of G protein33; butanol, a competitive substrate of PLD that reduces PLD-catalyzed production of phosphatidic acid34; and propranolol,35 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 G2/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 G2/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
transport processes was analyzed. As shown in Figure 7, PDMP significantly decreased specific HDL₃-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 Gα₁, Gα₂, and Gα₃ and Determination of the mRNA Expression Levels of These G-Protein Subunits in TD Fibroblasts**

To determine whether mutations in Gα₁, Gα₂, or Gα₃ 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-α and -β 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...
Influence of G Protein and PLD Signaling Modulators on Cell Cycle Distribution

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<th>Control Fibroblasts</th>
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<td>G2/M Phase, %</td>
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<td>100 ng/mL PTX</td>
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Control and TD fibroblasts were grown for 3 days in DMEM supplemented with 10% FCS and then incubated with or without (basal) the indicated substance for 24 hours in DMEM with 10% FCS, the cell cycle distribution was determined as described in Methods. Data for cells in the S and G2/M phases are expressed as percent of total cells. Results represent mean±SEM of 3 and 2 independent experiments in duplicate from fibroblasts of 3 control subjects and 2 TD patients, respectively.

*P<0.05 for treated vs untreated.

Discussion

The current study shows that cultured fibroblasts from patients with TD are characterized by an abnormal morphology and a markedly reduced in vitro growth compared with fibroblasts from control subjects. The reduced proliferation of TD fibroblasts is due to an impaired cellular response that cannot be explained by a lack of either well-known autocrine or paracrine growth factors or a reduced attachment of the initially seeded fibroblasts. Furthermore, by stimulating cells with EGF as well as PDGF-AB, it was demonstrated that the impaired cellular response was not restricted to a specific growth factor 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 G2/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 MNP s 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 retromediation of HDL particles, the processing of acylated LDL, and the transport of newly synthesized sphingomyelin and glucosylceramide to the plasma membrane 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 endocytotic 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,

![Figure 7. Effect of PDPMP on HDL3-mediated [3H]cholesterol and [31C]cholesterol efflux from control and TD fibroblasts. Control (open bar) and TD (filled bar) fibroblasts were labeled with [31C]cholesterol and [3H]cholesterol for 48 hours. The last 12 hours of pulse incubation were performed in the presence or absence of 40 μmol/L PDPMP. HDL3-mediated cholesterol efflux was determined as described in Methods. Each bar represents mean±SEM of 3 experiments, performed in duplicate, on fibroblasts from 3 control subjects and TD patients, respectively. *P<0.05 for treated.](image-url)
1998), other monensin effects besides those on the Golgi apparatus cannot be excluded.

Ceramide has been described as a negative regulator of the cell cycle\textsuperscript{22,26,41} 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{25} (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 reduction of the HDL\textsubscript{3}-mediated lipid efflux in control cells by PDMP, whereas the already reduced efflux in TD fibroblasts was not affected.

Impairment of G\textsubscript{i}-protein-mediated signal transduction (eg, activation of PI-PLC and PLD) was demonstrated to be relevant for the reduced HDL\textsubscript{3}-mediated lipid efflux of TD fibroblasts.\textsuperscript{7,11,13} Because PLD is also thought to participate in mitogenic signaling,\textsuperscript{23} the question arises as to whether defective G\textsubscript{i}-protein-mediated signaling is involved in the growth and cell cycle abnormalities of TD fibroblasts. We have demonstrated that neither the blockade of G\textsubscript{i} protein function with PTX\textsuperscript{35} nor the inhibition of phosphatidic acid generation by butanol\textsuperscript{14} was able to induce a G\textsubscript{2}/M-phase arrest or to elevate cellular ceramide levels in control cells. Also propranolol, a substance known to increase intracellular phosphatidic acid concentrations,\textsuperscript{40} was ineffective in normalizing the cell cycle distribution in TD cells. These findings support the following interpretations: (1) Together with a normal expression of transcripts for \alpha-subunits of the G\textsubscript{i} proteins, ARF1,\textsuperscript{44} ARF3,\textsuperscript{45} PITP-\alpha,\textsuperscript{46} and PITP\beta,\textsuperscript{47} all of which are known to be involved in PI-PLC– or PLD-mediated signal transduction, the inoperative G\textsubscript{i}-protein signaling in TD fibroblasts is likely due to functional downregulation rather than a structural defect. (2) The effect of ceramide on the cell cycle is unlikely to be mediated via inhibition of PLD. Downregulation of PLD- and PI-PLC– mediated signal transduction in TD fibroblasts may involve ceramide and other sphingolipids\textsuperscript{48} but can also be related to caveolae, because these special membrane domains are considered to be signal transduction centers and their formation depends on a functional Golgi apparatus.\textsuperscript{49,50} The involvement of caveolae in the pathophysiology of 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.

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