Discordant regulatory changes in monocrotaline-induced megalocytosis of lung arterial endothelial and alveolar epithelial cells

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Discordant regulatory changes in monocrotaline-induced megalocytosis of lung arterial endothelial and alveolar epithelial cells. Am J Physiol Lung Cell Mol Physiol 290: L1216–L1226, 2006. First published January 13, 2006; doi:10.1152/ajplung.00535.2005.—Monocrotaline (MCT) causes pulmonary hypertension in the rat by a mechanism characterized by megalocytosis (enlarged cells with enlarged endoplasmic reticulum and Golgi and a cell cycle arrest) of pulmonary arterial endothelial (PAEC), arterial smooth muscle, and type II alveolar epithelial cells. In cell culture, although megalocytosis is associated with a block in entry into mitosis in both lung endothelial and epithelial cells, DNA synthesis is stimulated in endothelial but inhibited in epithelial cells. The molecular mechanism(s) for this dichotomy are unclear. While MCTP-treated PAEC and lung epithelial (A549) cells both showed an increase in the “promitogenic” transcription factor STAT3 levels and in the IL-6-induced nuclear pool of PY-STAT3, this was transcriptionally inactive in A549 but not in PAEC cells. This lack of transcriptional activity of STAT3 in A549 cells correlated with the cytoplasmic sequestration of the STAT3 coactivators CBP/p300 and SRC1/NcoA in A549 cells but not in PAEC. Both cell types displayed a Golgi trafficking block, loss of caveolin-1 rafts, and increased nuclear Ire1α, but an incomplete unfolded protein response (UPR) with little change in levels of UPR-induced chaperones including GRP78/BiP. There were discordant alterations in cell cycle regulatory proteins in the two cell types such as increase in levels of both cyclin D1 and p21 simultaneously, but with a decrease in cdc2/cdk1, a kinase required for entry into mitosis. While both cell types showed increased cytoplasmic geminin, the DNA synthesis-initiating protein Cdt1 was predominantly nuclear in PAEC but remained cytoplasmic in A549 cells, consistent with the stimulation of DNA synthesis in the former but an inhibition in the latter cell type. Thus differences in cell type-specific alterations in subcellular trafficking of critical regulatory molecules (such as CBP/p300, SRC1/NcoA, Cdt1) likely account for the dichotomy of the effects of MCTP on DNA synthesis in endothelial and epithelial cells.

pulmonary hypertension; pyrrolizidine alkaloids; STAT3 signaling; unfolded protein response; cell cycle and DNA synthesis regulatory proteins

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function of this organelle resulting in the inhibition of entry into mitosis (25, 31, 47). We also reported, as would have been expected from the Golgi trafficking block, that the initial steps in the unfolded protein response appeared to have been triggered (for example, the presence of Ire1α in the nucleus) (31).

These observations raised several questions. Was the downstream transcriptional activity of PY-STAT3 maintained in megalocytosis? Were levels of known STAT3-induced promitogenic target proteins, such as cyclin D1, increased? Were levels of downstream effectors of the unfolded protein response (UPR; such as GRP78/BiP and other chaperones) enhanced? Unexpectedly, we now report that MCTP-treated cells are dysfunctional in downstream events in transcription, UPR, cell cycle, and DNA synthesis pathways. STAT3 transcriptional activity was interfered with in A549 cells because the critical coactivators (CBP/p300 and SRC1) were no longer in the nucleus and the UPR was incomplete in both cell types. In comparing megalocytotic lung endothelial with epithelial cells, there were both concordant and discordant molecular changes comparing megalocytotic lung endothelial with epithelial cells, the nucleus and the UPR was incomplete in both cell types. In downstream effectors of the unfolded protein response (UPR; such as GRP78/BiP and other chaperones) entry into mitosis is expected from the Golgi trafficking block, that the initial steps in the unfolded protein response appeared to have been triggered (for example, the presence of Ire1α in the nucleus) (31).

MATERIALS AND METHODS

Cell culture, growth, and fractionation. Human pulmonary type II-like alveolar epithelial cell line A549 was a gift from Dr. A. Ray, University of Pittsburgh School of Medicine. Cultures of primary bovine PAEC were a gift from Dr. S. Olson, New York Medical College, and were used between passage 4 and 20. Growth of A549 and PAEC in T-75 flasks, 100-mm plastic Petri dishes, or six-well plates were carried out as previously reported (31, 35, 45, 47).

For use in cell culture, MCTP was prepared from MCT (purchased from TransWorld Chemicals, Rockville, MD) using the procedure of Mattocks et al. (27). By mass analyses, ~30–50% of the input monocrotaline was converted to the pyrrolic derivative (data not shown). MCTP was stored in small aliquots in dimethylformamide (DMF) at −80°C, diluted to the required concentration in DMF just before use and added directly to the cultures with gentle swirling. Control cultures received equivalent volume of DMF. Recombinant human IL-6 was purchased from R & D Systems (Minneapolis, MN).

Just subconfluent A549 and PAEC cultures in six-well or 100-mm plates plated 1 day earlier received either DMF alone (0.4% vol/vol) or MCTP in DMF (using the equivalent of 100 or 200 μM MCT with the fractional conversion to MCTP indicated above; see Refs. 31, 47). Typically, megalocytosis developed within 1–2 days and the cultures were used either at 2 or 4 days after MCTP. For immunofluorescence assays, the cultures were fixed using the cold-paraformaldehyde-2-phenylindole (DAPI). Images were collected using a Leitz epifluorescence microscope system equipped with a black-and-white CCD camera and then rendered in pseudocolor. All data within each experiment were collected at identical imaging settings and images were subjected to iterative deconvolution using the National Institutes of Health (NIH) Image J software.

Western blot analyses of proteins. Western blotting was carried out using 4–20% gradient polyacrylamide gels (Criterion gels, Bio-Rad Laboratories, Hercules, CA) under reducing and denaturing conditions as previously summarized (35, 45, 46). Proteins were detected using a chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, UK). Routinely, multiple exposures were collected so as to ensure data capture within the linear range of the Kodak BioMax film used. Each analysis was replicated at least twice and data were quantitated using the NIH Image J software.

35S-methionine pulse-chase experiments. A549 cell cultures in 100-mm plates were washed with PBS and replenished with serum-free and methionine-free DMEM (1 ml/culture) containing 35S-methionine (30 μCi/ml; sp. activity >1,000 Ci/mmol, MP Biomedical, Irvine, CA). After 1 h, the culture medium was changed to methionine (1 mM)- and serum-containing DMEM and chased for another 3 or 19 h. The cultures were harvested by preparing the Brij-58-Sup fraction. Protein-matched aliquots of respective control and MCTP-treated cell extracts were then precleared using Pansorbin (Calbiochem, La Jolla, CA) and immunoprecipitated using respective rabbit polyclonal antibodies to STAT3, cyclin D1, and p21 using the method of Grieninger et al. (11). The immunoprecipitates were analyzed by SDS-PAGE and autoradiography (11).

IL-6-responsive reporter/luciferase construct assays. Transfections into A549 cell cultures in six-well plates, in triplicate wells for each experimental variable, were carried out 1 day after MCTP treatment using the lipofectamine reagent (Polyfect, QIAGEN, Valencia, CA) and the manufacturer’s protocol. Briefly, the reporter/luciferase construct p950M4-luciferase which contains four copies of the STAT3-binding DNA element from the human angiotensinogen promoter (a gift from Dr. A. Kumar, Department of Pathology, New York Medical College) (16, 47) or pSTAT3-luciferase which contains four copies of the STAT3 response element from the rat α2-macroglobulin gene (a gift from Dr. D. Levy, Department of Pathology, New York University School of Medicine) (33) were transfected into A549 and PAEC cells (250–500 ng/well) together with the constitutive β-galactosidase expression plasmid pCH110 (50 ng/well). After incubation at 37°C for another 20–24 h, the cultures were treated with IL-6 (10 ng/ml) for 6 h and the cells were harvested in 300-μl lysis buffer (Promega Biotech, Madison, WI). Fifty-microliter aliquots of the clarified extracts were used to assay luciferase and β-galactosidase activity using respective assay kits/reagents from Promega Biotech and Roche Applied Science (Indianapolis, IN), respectively, and the manufacturer’s protocols. The luciferase activity was normalized with reference to the β-galactosidase activity in the extract and expressed in terms of that in the MCTP-free, IL-6-free controls.

Antibody reagents. Rabbit antibodies to STAT3, PY-STAT3, Ire1α, XBP1, ATF6, PERK, cyclin D1, cyclin D3, cyclin E, cyclin A, p21, PCNA as well as murine mAb to PY-STAT3, XBP1, Hsc70, cyclin D1, cyclin B1, cdc2, and nonimmune isotype-matched murine mAb were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit pAb to p-eIF2α was obtained from Stressgen Biotechnologies (BC, Canada). Murine mAbs to STAT3, STAT1, GM130, BIP (GRP78), Hsp90, and Hsp75 were purchased from BD Biosciences (Transduction Laboratory, San Diego, CA).

Statistical analyses. These were carried out using the two-tailed Student’s t-test and Microsoft Excel software.
RESULTS

Dysfunctional STAT3 signaling in MCTP-induced megalocytosis. We previously reported an inverse relationship between MCT-induced disruption of cave-1 rafts on the one hand and “hyperactivation” of IL-6/STAT3 signaling and increased DNA synthesis on the other hand in PAEC at the single cell level in vivo and in cell culture (25, 47). In the earlier studies, STAT3 “signaling” was assayed in terms of enhancement of STAT3 Tyr phosphorylation using immunohistochemical or Western blotting techniques and by STAT3-specific DNA binding in nuclear extracts. With the observation that MCTP-treated type II-like alveolar epithelial cells (A549) also showed a loss of cave-1 but an inhibition of DNA synthesis (31, 47), we asked whether sustained IL-6/STAT3 signaling was also observed in epithelial cells.

In addressing this question, we prepared cytoplasmic and nuclear fractions using a detergent-based approach [which gave a Brij-58-soluble cytoplasmic fraction, a DOC/Tween 40

![Western blot assays](image1)

**Fig. 1.** Dysfunctional IL-6/PY-STAT3 signaling in megalocytosis. A: BPAEC and A549 cultures in 100-mm plates were either left untreated (4 cultures) or treated with MCTP (8 cultures). Four days later, half of these were exposed to IL-6 for 30 min (2 MCTP-free controls and 4 MCTP-treated cultures). The cells were scraped into 0.25 M sucrose buffer containing 0.5% Brij-58 (1 ml per group with the same aliquot used to serially harvest cultures within the one experimental group) followed by separation into a supernatant (“Brij-Sup”) and pellet fraction by centrifugation. The Brij-pellet was further washed using isotonic buffer containing 0.5% DOC-Tween 40 followed by separation into a DOC-Sup fraction and the washed nuclear pellet. Western blot analyses for STAT3 and PY-STAT3 were carried out using matched amounts of total protein within each set of four lanes corresponding to the Brij-Sup, DOC-Sup, and DOC-pellet fractions using the untreated control samples (first lane in each set) that match the respective compartments within each set. B: untreated and MCTP-treated (4 days) A549 cultures were transferred to methionine- and serum-free medium and labeled with $^{35}\text{S}$-methionine (30 μCi/ml) for 1 h followed by a chase for either 3 or 19 h in methionine-containing medium. Labeling of STAT3 or cyclin D1 in protein-matched aliquots of the Brij-Sup from such cultures was evaluated by immunoprecipitation, SDS-PAGE, and autoradiography. C: assays for IL-6-induced transcriptional activity in untreated and MCTP-treated PAEC and A549 cultures were carried out using the p950M4-luciferase or the pSTAT3-luciferase reporter constructs. Cultures were first treated with MCTP and 1 or 2 days later were transfected with the respective reporter constructs together with the pCH110 constitutive $\beta$-galactosidase construct. Basal and IL-6-induced activation of the luciferase constructs was assayed 1 day after transfection as described in MATERIALS AND METHODS. Luciferase activity, normalized to $\beta$-galactosidase activity, is expressed in terms of that in the IL-6-free MCTP-untreated control cultures (means ± SE). *P < 0.01 in comparison with the respective MCTP-free cultures. NS, not significant (P > 0.05) in comparison with the respective MCTP-free cultures.
cytoplasmic wash fraction (which includes the outer nuclear membrane and a nuclear fraction)] (15, 38, 44) taking care to harvest cells in monolayer cultures directly into the Brij-58 lysis buffer so as to avoid any loss of cytosol (control experiments showed that scraping cells first into PBS followed by pelleting of cells led to loss of at least half of the cytosolic STAT3). The Western blotting data in Fig. 1A (BPAEC) confirm the enhancement of the nuclear pool of STAT3 and PY-STAT3 in megalocytic endothelial cells. Additionally, when care was taken to avoid losses of soluble STAT3, there was a clear (2-fold) increase in bulk STAT3 levels in the cytoplasm after MCTP. Figure 1A (A549) confirms the increase in bulk cytoplasmic STAT3 (5-fold) in lung epithelial cells after MCTP, as well as enhancement of IL-6-induced PY-STAT3 signaling into the nucleus. The increase in bulk cytoplasmic STAT3 levels was a general property of megalocytic cells in that it was also observed in hepatocytes (Hep3B) and breast carcinoma cells (MCF-7) (data not shown). 

35S-methionine pulse-chase experiments showed that this increase was due to an increase in the rate of synthesis of STAT3 protein and that, as in control A549 cultures, the STAT3 protein itself was quite stable during a 20-h chase (Fig. 1B). In comparison, while cyclin D1 synthesis was also enhanced after MCTP, this protein, as expected, was unstable in both control and MCTP-treated lung epithelial cells during the chase (Fig. 1B).

Immunofluorescence studies (Fig. 2) provided data consistent with Western blotting. In A549 cells, IL-6 treatment increased STAT3 and PY-STAT3 in the nucleus. However, in some megalocytic A549 cells STAT3 remained sequestered in a perinuclear cytoplasmic location partially overlapping with the cis-Golgi marker GM130. The Golgi organelle was enlarged/circumnuclear in megalocytosis, confirming our previous observations (31, 47). IL-6-induced enhancement of nuclear STAT3 and PY-STAT3 pools as well as Golgi enlargement data were also observed in PAEC using immunofluorescence methods (data not shown).

In light of the above cell fractionation, Western blotting, and immunofluorescence data, we expected a commensurate enhancement of PY-STAT3 transcriptional activity in both cell types. To the contrary, the data in Fig. 1C show that both basal and IL-6-induced STAT3-reporter-luciferase activity using two different reporter constructs was markedly inhibited in megalocytic A549 cells but not in PAEC. The underlying mechanism accounting for this dichotomy emerged in immunofluorescence studies of the transcriptional coactivators that are required for PY-STAT3-mediated transcriptional activity (Fig. 3). In megalocytic A549 cells, the critical STAT3

![Image](http://ajplung.physiology.org/)

**Fig. 2.** Intact IL-6-induced STAT3 and PY-STAT3 signaling in control and megalocytic A549 cells using an immunofluorescence assay. A549 cells in 6-well plates were treated with dimethylformamide or exposed to MCTP. Four days later, respective cultures were treated with IL-6 for 30 min and fixed using the cold paraformaldehyde-Triton method. STAT3, PY-STAT3, and GM130 immunofluorescence was evaluated as indicated. Scale bar = 50 μM.
coactivators CBP/p300 (a histone acetyl transferase) and SRC1/NcoA (another histone acetyl transferase and a transcription start site scaffolding protein), which are normally nuclear in location (9, 34, 37, 40), were largely sequestered in the cytoplasm in MCTP-treated cells (Fig. 3B). While some cells in control cultures displayed cytoplasmic CBP and some cells after MCTP displayed nuclear SRC1 suggesting a level of cellular heterogeneity within each culture, overall, there was a marked cytoplasmic sequestration of these critical coactivators. In contrast, both these coactivators remained largely nuclear in MCTP-treated endothelial cells (Fig. 3A). Thus STAT3 signaling was dysfunctional in megalocytotic epithelial cells but not in endothelial cells because the downstream coactivators were in the wrong cellular compartment in the former but not the latter cell type. In as much as coactivators such as CBP/p300 and SRC1/NcoA are also utilized as bridging factors or scaffolds to recruit the transcriptional machinery and RNA polymerase II by other DNA-binding proteins (9, 34, 37, 40), the data in Fig. 3B suggest a broad transcriptional dysfunction in megalocytotic lung epithelial cells.

Incomplete UPR in MCTP-induced megalocytosis. We previously reported that treatment of A549 cells with MCTP led to an increase in the nuclear pool of the endoribonuclease Ire1α and the chaperone GRP58, with enhanced circumnuclear distribution of PERK kinase and the chaperone PDI (31). Nuclear Ire1α is known to act as an endoribonuclease which leads to production of an mRNA for the transcriptionally active form of XBP1 (22, 36, 56). PERK phosphorylates the eukaryotic initiation factor 2α (eIF2α) thus attenuating protein translation (3, 12). The third arm of the UPR is the ATF6 pathway. Accumulation of unfolded proteins in the ER leads to a translocation of ATF6 from the ER to the Golgi and subsequent sequential cleavage by the actions of S1P and S2P proteases (reminiscent of the SREBP) (6, 32, 48). The released cytosolic fragment of ATF6 then translocates to the nucleus and activates gene transcription. The end result of the UPR is an increase in the levels of chaperone proteins which target misfolded proteins toward degradation: the chaperones GRP78/BiP, Hsc70, Hsp90, Hsp75, and PDI (22, 36, 56).
Figure 4A shows that Ire1α is nuclear in both megalocytic epithelial and endothelial cells indicating that the proximal sensor for the UPR is activated in both cell types. However, this is not accompanied by an increase in nuclear XBP1 or ATF6; both ATF6 and XBP1 are trapped in a circumnuclear cytoplasmic compartment, partially colocalizing with GM130 (Figs. 4B and 5). With respect to the PERK pathway, there is a clear increase in p-eIF2α, especially in the nucleus (Fig. 4B).

Fig. 4. Activation of the unfolded protein response (UPR) in megalocytosis. Control and megalocytic (4 days after MCTP) cultures of BPAEC and A549 cells in 6-well plates were evaluated for the proximal sensors and distal outcome of the UPR using immunofluorescence assays as indicated. Scale bar = 50 μM.
Overall, there was little change in cytoplasmic BiP (Figs. 4B and 5).

Figure 5A is a summary of Western blotting data derived from A549 cells which confirm the increase in XBP1 forms (the “spliced” active form as assayed using an anti-XBP1 mAb, a complex ~200 kDa and the inactive form of 31 kDa as assayed using an anti-XBP1 pAb), but all in the cytoplasm (Brij-Sup). Similarly, the predominant ATF6 form is also observed in the cytoplasm (DOC-sup). Figure 5B summarizes data showing little change in GRP78/BiP, Hsc70, Hsp90, PDI, and Hsp75 in megalocytotic cells in different subcellular compartments. Additionally, in endothelial cells, too, there was little change in XBP1 or ATF6 (both of which remained largely cytoplasmic) and little change in levels of chaperone proteins (data not shown). Thus, although some of the pathways triggered in the UPR were activated (Ire1/H9251 was nuclear in both cell types), the UPR was incomplete in that there was little change in levels of chaperone proteins.

**Discordant changes in cell cycle and DNA synthesis regulatory proteins.** We began by analyzing “promitogenic” cell cycle regulatory proteins such as cyclin D1 that are supposed to be increased by STAT3 (reviewed in Ref. 4). Contrariwise, the increase in p-eIF2α in megalocytosis (Fig. 4B) would have predicted a decrease in cyclin D1 (3, 12).

As a frame of reference for these studies, while megalocytotic endothelial cells showed increased and ongoing DNA synthesis, epithelial cells (lung A549, liver Hep3B, and breast MCF7) do not. Nevertheless, previous investigators focusing on endothelial cells and believing that the megalocytotic phenotype reflected only a G2/M block per se focused on analyses of G2-phase cyclins and reported increases in cyclins B1 and A and in p21 with an increase in cdc2 and triphosphorylated inactive p-cdc2 (49, 50, 52). In an earlier report, we pointed out that in all cell types investigated, megalocytosis was characterized by an inhibition of entry into M (47). However, we reported an increase in the formation of complexes between p-GM130 with cdc2 (hypothesized to be required for Golgi fragmentation and entry into M). The data in Fig. 6 show an increase in the promitogenic G1 or S phase cyclins D1, D3, and E (including in the nucleus in the case of D3 and E) in megalocytosis of epithelial A549 cells together with an increase in the inhibitory p21 and a decrease in cyclin B1. The increased cyclin D1 was, however, sequestered in the cytoplasm, away from the site of its action (Fig. 6 and immunofluorescence data in Fig. 7). In BPAEC, there were increases in free cyclin D1 and D3 in the cytoplasm, an increase in

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Fig. 5. Incomplete UPR in megalocytosis. Control and megalocytotic (4 days after MCTP) cultures of A549 cells in 100-mm plates were harvested into the Brij-Sup and Brij-Pellet or Brij-Sup, DOC-Sup, and nuclear pellet fractions as indicated in Fig. 1 and MATERIALS AND METHODS. A and B: composite summary of Western blot analyses for XBP1 and ATF6 forms and for various chaperone proteins (using matched amounts of total protein within each set compared; see Fig. 1 legend).

Fig. 6. Discordant changes in cell cycle and DNA synthesis regulatory proteins in megalocytosis. Control and megalocytotic (4 days after MCTP) cultures of BPAEC and A549 cells in 100-mm plates were harvested into the Brij-Sup, DOC-Sup, and nuclear pellet fractions as indicated in Fig. 1 and MATERIALS AND METHODS. A and B: composite summary of Western blot analyses for respective cell cycle and DNA synthesis regulatory proteins (using matched amounts of total protein within each set compared; see Fig. 1 legend).

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high-molecular-weight complexes of D3 but not of D1, a decrease in cyclin E and PCNA, but an increase in p21 and cyclin B1. In both cell types, there was a decrease in bulk cdc2/cdk1. Our data confirm the previous observations of Wilson et al. (52) in human PAEC treated with MCTP for 2 days with respect to cyclin B1 and p21, but, using Western blotting techniques, show a marked decrease in bulk cdc2/cdk1, a kinase crucial to entry of cells into M (23, 24).

With respect to molecules regulating DNA synthesis, some of the changes were paradoxical: by Western blotting there was little change in PCNA in epithelial cells (which show an inhibition of BrdU labeling), but there was a reduction in BPAEC (which show an increase in BrdU labeling; Fig. 6). A mechanism for the discordance in DNA synthesis phenotype between endothelial and epithelial cells emerged in immunofluorescence studies which showed that while the DNA syn-

![Fig. 7. Discordant changes in cell cycle and DNA synthesis regulatory proteins in megalocytosis in PAEC and A549 cells. Control and megalocytotic (4 days after MCTP) cultures of PAEC and A549 cells in 6-well plates were evaluated for the subcellular localization of cyclin D1, geminin, and Cdt1 as indicated. Nuclei were demarcated using DAPI. Scale bar = 50 μM.](image-url)
thesis-initiating protein Cdt1 was sequestered in the cytoplasm in megalocytotic A549 cells, it remained largely nuclear in PAEC despite the fact that in both cell types the Cdt1-sequestering protein geminin was markedly increased in the cytoplasm (43, 55) (Fig. 7). Thus the data with respect to Cdt1 localization provide a molecular basis for understanding the discordant DNA synthesis phenotype between MCTP-treated lung endothelial and epithelial cells.

**DISCUSSION**

The “megalocytosis” phenotype produced by pyrrolizidine alkaloids was first reported in 1942 (13, 14) and the term was coined in 1955 (5). Specifically, for over four decades, the MCT-treated juvenile male rat has been extensively investigated as a model for PH (reviewed in Ref. 47). In this model, at the cellular level, there is megalocytosis of the pulmonary arterial endothelial, arterial smooth muscle cells, and alveolar type II epithelial cells. In these cell types, megalocytosis is characterized by a block in entry into M despite continuing cellular “growth.” In exploring the underlying mechanisms leading to megalocytosis, we discovered that there was a block in trafficking through the Golgi resulting in disruption of plasma membrane cave-1 rafts and increased STAT3 and ERK1/2 “promitogenic” signaling. Nevertheless, the mechanisms that underlie the dichotomy that DNA synthesis is stimulated in megalocytotic endothelial cells (resulting in tetraploid and hypertetraploid cells) but inhibited in epithelial cells (20, 21, 25, 31, 41, 47, 50, 54) remained obscure.

The present data (summarized in Table 1) show that despite evidence for activation of the IL-6/STAT3 pathway in megalocytotic epithelial cells, including a fivefold increase in bulk cell labeling (Table 1). Thus the data with respect to Cdt1 localization provide a molecular basis for understanding the discordant DNA synthesis phenotype between MCTP-treated lung endothelial and epithelial cells.

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<tr>
<th>Protein/Activity</th>
<th>Function</th>
<th>Lung Cell Type</th>
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<tbody>
<tr>
<td>Entry into mitosis^a</td>
<td>Cell cycling</td>
<td>Endothelial (PAEC)</td>
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<tr>
<td>BrdU labeling^a</td>
<td>DNA synthesis</td>
<td>Predom. nuclear</td>
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<tr>
<td>Cdt-1</td>
<td>Initiates DNA synthesis</td>
<td>Predom. cytopl.</td>
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<tr>
<td>Geminin</td>
<td>Sequesters Cdt-1</td>
<td>Nuclear</td>
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<tr>
<td>PCNA</td>
<td>Initiates DNA synthesis</td>
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<tr>
<td>STAT3</td>
<td>Transcription factor</td>
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<tr>
<td>PY-STAT3</td>
<td>Activated STAT3</td>
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<tr>
<td>pSTAT3-fac</td>
<td>Gene activation</td>
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<tr>
<td>SRC1/NcoA</td>
<td>STAT3 coactivator/HAT^b</td>
<td>Down from nucleus, ↑ in cytopl.</td>
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<tr>
<td>Ire1α</td>
<td>Splices XBP1 intron, activates UPR</td>
<td>Nuclear</td>
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<tr>
<td>UPR</td>
<td>↑ Chaperones; ↑ ER, Golgi structure</td>
<td>Complete</td>
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<tr>
<td>Cyclin D1</td>
<td>↑ In G1, ↓ in S</td>
<td>↑ Cytopl. seq. ^e</td>
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<tr>
<td>Cyclin D3</td>
<td>↑ In G1</td>
<td>↑ In cytopl. and ↑ nuc. complex</td>
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<tr>
<td>Cyclin E</td>
<td>↑ In G1, initiates S</td>
<td>From cytopl.</td>
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<tr>
<td>Cyclin A</td>
<td>↑ In S and G2</td>
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<tr>
<td>Cyclin B1</td>
<td>↓ In G1, ↑ in G2, initiates M</td>
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<tr>
<td>Cdc2</td>
<td>↓ In G1, ↑ in G2, initiates M</td>
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<tr>
<td>p21</td>
<td>Inhibits S</td>
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^aFrom Refs. 31 and 47, ^bHistone acetyl transferase; ^eperinuclear sequestration; ↓, decreased levels; ↑, increased levels; ↔, no change; ^e, not detectable in either control or MCTP-treated cells in current experiments; data not shown. Cytopl, cytoplasmic; seq., sequestration; PAEC, pulmonary arterial endothelial cells. From Wilson and colleagues (Refs. 49, 50, and 52): in PAEC ↑ cdc2 and p-cdc2, ↑ cyclins B1 and A, ↑ p21 From Shah et al. (Ref. 47): ↑ p-GM130 and ↑ binding of cdc2 to p-GM130.
teins and growth factor receptors (as examples, cav-1, PECAM-1, and E-cadherin), as well as to selective defects in nucleo-cytoplasmic shuttling of transcription and DNA synthesis regulatory proteins (as examples CBP, SRC1, cyclin D1, Cdt1, and geminin).

Blocking of ER to Golgi trafficking is known to activate the UPR (32). As MCTP causes a phenotype indicative of such a block (“proliferation” of the ER and Golgi as investigated by electron microscopy of lung tissue and of cells in culture; Refs. 29, 51, 54), we hypothesized that MCTP might also activate the UPR. Typically, the UPR leads to enhanced synthesis of ER-lumenal chaperone proteins, increased production of structural proteins that constitute the ER, and attenuated translation of defective and misfolded proteins. Our data show that while the proximal sensors of the UPR were activated in megacytosis (nuclear Ire1α and the PERK/p-eIF2α pathway), there was little increase in nuclear pools of either XBP1 or ATF6. Both these transcriptional arms of the UPR were trapped in perinuclear structures, partially colocalizing with the cis-Golgi marker GM130. Parenthetically, ATF6 is ordinarily localized in the ER membrane and has to traffic to the Golgi for cleavage and activation (6, 32, 48). Thus, if there is a block in trafficking from ER to the Golgi and of transit through the Golgi, this trafficking of ATF6 would also be blocked accounting for the perinuclear trapping of ATF6. Furthermore, the cytoplasmic perinuclear trapping of XBP1, which is typically considered to be a nuclear transcription factor, is consistent with the detection of 200-kDa complexes containing XBP1 in the Brij-58-soluble fraction. Other investigators have also noted perinuclear/cytoplasmic accumulations of XBP1 in osteoblasts and human breast cancer (8, 57) but the functional consequences of this localization have remained elusive. Thus, with respect to megacytosis and the UPR, while the proximal sensors were activated in both endothelial and epithelial megacytotic cells, several of the downstream events were defective and incomplete. There was little increase in levels of chaperone proteins as well as several of the downstream events were discordant. STAT3 signaling was interfered with in epithelial cells but not endothelial cells likely because the DNA synthesis initiating protein Cdt1 was sequestered in the cytoplasm in the former cell type. However, in both cell types the UPR was incomplete, and the overall alterations in cell cycle regulatory molecules were self-contradictory. Thus the ordinary descriptions of cell cycle progression from G1 to S to G2 do not appear to apply in megacytosis. The data suggest that MCTP-induced megacytosis is a phenotype resulting from interference with subcellular trafficking mechanism (tol/through the Golgi and nucleo-cytoplasmic shuttling) of critical transcription, cell cycle, and DNA synthesis regulatory molecules.

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REFERENCES


