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

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Mukhopadhyay, Somshuvra, and Pravin B. Sehgal. 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 endothelium but inhibited in epithelial cells. The molecular mechanisms(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 Ire1a, 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. 

The monocrotaline (MCT)-treated rat has been used extensively over the last four decades as a convenient model to investigate the pathogenesis of pulmonary hypertension (PH) (17, 29, 54; reviewed in Refs. 25, 31, 47). A single subcutaneous injection of this pyrrolizidine plant alkaloid into a juvenile male rat results in the development of irreversible PH 10 to 14 days later. The injected MCT is bioactivated by the cytochrome P-450 pathway to its pyrrolic derivative (MCTP) in the liver with complete clearance of all metabolites from the body in less than 1 day (reviewed in Refs. 31, 47). The active metabolite, MCTP, has a half-life of ~3 s in aqueous medium (26) and thus primarily affects the first vascular bed, the pulmonary arterial tree, it encounters in traveling from its origin in the liver. The major cellular effect seen after MCTP treatment in vivo and in cell culture is the development of “megalocytosis” or “karyocytomegaly” characterized by increased cell size with increase in the endoplasmic reticulum (ER) and Golgi stacks, ongoing DNA synthesis in lung arterial endothelial cells but not alveolar epithelial cells but with a cell cycle arrest (characteristically a block in entry into mitosis) (1, 2, 5, 7, 13, 14, 20, 21, 28, 30, 39, 41, 50–52, and 54; reviewed in Refs. 31, 47 and citations therein). Such cells have been described as “growing but not dividing” (1; reviewed in Ref. 47). In the lung in vivo, megalocytotic changes are seen in the pulmonary arterial endothelial and smooth muscle cells and in alveolar type II epithelial cells (29, 41, 42, 51, 54). Numerous investigators, including this laboratory, have recapitulated the development of megalocytosis in each of these three lung cell types in culture within 24–48 h after a single transient exposure to MCTP (20, 21, 25, 31, 41, 47, 50, 54). However, there are cell type-specific differences with respect to the status of DNA synthesis: while megalocytic pulmonary arterial endothelial cells (PAEC) show an increase in nuclear labeling index by BrdU, alveolar epithelial cells show an inhibition (20, 21, 25, 31, 41, 47, 50, 54). The specific molecular mechanisms leading to megalocytosis in different lung cells and why the DNA synthesis phenotype is different in endothelial cells compared with epithelial cells remain largely obscure.

We previously reported that in both endothelial and epithelial cells, exposure to MCTP led to disruption of plasma membrane caveolin-1 (cav-1)-containing rafts (25, 31, 47). In endothelial cells in vivo and in cell culture, we reported an inverse relationship between cav-1 raft disruption and hyperactivation of “promitogenic” STAT3 and ERK1/2 signaling as evaluated in biochemical assays (Tyr-phosphorylation and DNA-binding assays for activated STAT3 and Ser-phosphorylation of ERK1/2). This promitogenic signaling was associated with increased DNA synthesis in megalocytic endothelial cells as assayed by BrdU labeling and nuclear localization of PCNA. Subsequently, we showed that the disruption of cav-1 rafts was likely the result of a block of trafficking of cav-1 through the Golgi. More generally, in both endothelial and epithelial megalocytotic cells there was an enlargement of the Golgi compartment (the “Golgi blockade hypothesis”) (25, 31, 47). We and, prophetically, Afzelius and Schoental in 1967 (1) suggested that the Golgi blockade produced by pyrrolizidine alkaloids included the disruption of the mitosis-sensor
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 in cell cycle and DNA synthesis regulatory molecules consistent with the divergent DNA synthesis phenotype in the two cell types.

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-Triton X-100 procedure described earlier (31, 47). For biochemical assays (Western blots and immunoprecipitation), cultures were harvested within each 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-1% Tween 40 followed by separation into a DOC-Sup fraction and the washed nuclear pellet (15, 38). The Brij-Sup and DOC-Sup represent cytoplasmic fractions (the latter includes the outer nuclear membrane; Ref. 15), whereas the DOC pellet represents the nuclear fraction (with intact inner nuclear membrane; Ref. 15). There is little cytoplasmic contamination in the DOC pellet (15, 38, 44).

Double-label immunofluorescence studies. These were carried out on A549 and PAEC cells in six-well plates fixed using the cold paraformaldehyde-Triton X-100 procedure (31, 47) and respective rabbit polyclonal and murine monoclonal antibodies in various combinations and corresponding AlexaFluor 488 or AlexaFluor 594 secondary antibodies (Molecular Probes, Eugene, OR) as indicated in the individual experiments. Nuclei were demarcated using 4′,6-diamidino-2-phenylindole (DAPI). Images were collected using a Leitz epifluorescence microscopy 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. Ray, 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 Biotechnology 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, XBPI, ATF6, PERK, cyclin D1, cyclin D3, cyclin E, cyclin A, p21, PCNA as well as murine mAb to PY-STAT3, XBPI, 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-ERK2 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

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 transfected to methionine- and serum-free medium and labeled with 35S-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 transcripational 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 β-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 β-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 megalocytotic 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 megalocytotic 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 megalocytotic 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 megalocytotic 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 megalocytotic A549 cells, the critical STAT3

Fig. 2. Intact IL-6-induced STAT3 and PY-STAT3 signaling in control and megalocytotic 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).

Fig. 3. MCTP-induced megalocytosis disrupts the nuclear localization of the STAT3 coactivators CBP and SRC1 in A549 cells but not pulmonary arterial endothelial cells (PAEC). Control and megalocytotic (4 days after MCTP) cultures of PAEC or A549 cells in 6-well plates were evaluated for the subcellular localization of CBP and SRC1 in immunofluorescence assays. Nuclei were demarcated using DAPI. Scale bar = 50 μM.
Figure 4A shows that Ire1α is nuclear in both megalocytotic 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 megalocytotic (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 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 megalocytic 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 megalocytic 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...
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 immuno-fluorescence 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.](http://ajplung.physiology.org/)

http://ajplung.physiology.org/
thesis-initiating protein Cdt1 was sequestered in the cytoplasm in megalocytic 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 megalocytic 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 megalocytic epithelial cells, including a fivefold increase in bulk STAT3 levels, the downstream transcriptional function of PY-STAT3 in the nucleus was defective because the coactivators which recruit RNA polymerase (CBP/p300 and SRC1/ATF6) were either unchanged or sequestered in the cytoplasm. Thus the UPR was incomplete in both cell types in that levels of chaperone proteins were not increased. Megalocytic endothelial and epithelial cells showed both concordant and discordant changes in cell cycle and DNA synthesis regulatory proteins. Strikingly, the DNA synthesis-initiating protein Cdt1 was sequestered outside the nucleus in A549 cells in which DNA synthesis is inhibited but not in PAEC in which DNA synthesis is stimulated. Other regulatory proteins such as cyclin D1 and geminin were sequestered in the cytoplasm in both cell types. Overall, the alterations of cell cycle regulatory proteins were contradictory (as examples the “proproliferative” “G1” cyclins D1, D3, and E and the inhibitory regulator p21 were all increased). Despite these discordant alterations, both cell types showed reduced entry into M perhaps because of a reduction in both cell types of bulk cdc2/cdk1 kinase (which is required for Golgi fragmentation and transit into M). Taken together, the present data reveal that the MCTP-induced megalocytic cell represents a novel state with respect to the cell cycle for which the customary concept of a G1 to S to G2 progression or a G2/M block per se (20, 21, 49, 50, 52) does not appear to apply. There are also clear distinctions in the underlying molecular events in megalocytic endothelial and epithelial cells (Table 1).

A second theme which emerges from our studies is that MCTP disrupts the flow of critical regulatory molecules between and among different subcellular compartments resulting in defective trafficking. Our data point to defects in ER to Golgi and trans-Golgi trafficking of membrane-associated pro-

### Table 1. Discordant changes in transcription, cell cycle, and DNA synthesis regulatory proteins in megalocytosis

<table>
<thead>
<tr>
<th>Protein/Activity</th>
<th>Function</th>
<th>Endothelial (PAEC)</th>
<th>Epithelial (A549)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cdt-1</td>
<td>Initiates DNA synthesis</td>
<td>Predom. nuclear</td>
<td>Nuclear; cytopl. seq.</td>
</tr>
<tr>
<td>Geminin</td>
<td>Initiates DNA synthesis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STAT3</td>
<td>Transcription factor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PY-STAT3</td>
<td>Activated STAT3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSTAT3-fac</td>
<td>Gene activation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBP/p300</td>
<td>STAT3 coactivator/HAT&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
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<tr>
<td>SRC1/NcoA</td>
<td>STAT3 coactivator/HAT&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ire1α</td>
<td>Splices XBP1 intron, activates UPR</td>
<td>Incomplete</td>
<td>Incomplete</td>
</tr>
<tr>
<td>UPR</td>
<td>Chaperones; ↓ ER, Golgi structure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>↑ In G1, ↓ in S</td>
<td>↑ From cytopl.</td>
<td>↑ From cytopl.</td>
</tr>
<tr>
<td>Cyclin D3</td>
<td>↑ In G1</td>
<td>↑ In cytopl. and ↑ nuc. complex</td>
<td>↑ In cytopl. and nucleus</td>
</tr>
<tr>
<td>Cyclin E</td>
<td>↑ In G1, initiates S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin A</td>
<td>↑ In S and G2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin B1</td>
<td>↑ In G1, ↑ in G2, initiates M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cdc2</td>
<td>↑ In G1, ↑ in G2, initiates M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p21</td>
<td>Inhibits S</td>
<td>↑ Predom. cytopl.</td>
<td>↑ Predom. cytopl.</td>
</tr>
</tbody>
</table>

<sup>a</sup>From Refs. 31 and 47.<br><sup>b</sup>Histone acetyl transferase; <sup>c</sup>perinuclear sequestration; <sup>d</sup>not detectable in either control or MCTP-treated cells in present experiments; <sup>e</sup>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.
tein and growth factor receptors (e.g., caveolin-1, PECAM-1, and E-cadherin), as well as to selective defects in nucleo-cytoplasmic shuttling of transcription and DNA synthesis regulatory proteins (e.g., 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 megacellosis (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 epithelial cells but not in 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 megacellosis. The data suggest that MCTP-induced megalocytosis is a phenotype resulting from interference with subcellular trafficking mechanism (to/through the Golgi and nucleo-cytoplasmic shuttling) of critical transcription, cell cycle, and DNA synthesis regulatory molecules.

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REFERENCES


19. Lame MW, Jones DA, Wilson DW, and Segall HJ. Monocrotaline pyrrole targets proteins with or without cysteine residues in the cytosol and membranes of human pulmonary artery endothelial cells. Proteomics In press.


