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Alterations of growth factor transcripts in rat lungs during development of monocrotaline-induced pulmonary hypertension

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Abstract—Although pathologic and hemodynamic changes in monocrotaline (MCT)-induced pulmonary hypertension have been studied extensively, relatively little is known about the inter- and intracellular signaling mechanisms underlying such alterations. As a first step to delineating signaling mechanisms governing adverse structural alterations in the hypertensive lungs, we examined changes in the steady-state levels of mRNAs encoding several growth factors including transforming growth factors (TGF), platelet-derived growth factors (PDGF), vascular endothelial cell growth factor (VEGF) and endothelin (ET) as a function of time in MCT-induced pulmonary hypertension in rats. These studies demonstrated a very diverse pattern of growth factor gene expression in response to MCT administration. In general, alterations in the steady-state levels of mRNAs encoding the growth factors preceded the onset of MCT-induced pulmonary hypertension. TGF-β1, -β2 and -β3 transcripts were seen to be elevated, whereas that of TGF-α and PDGF-A remained unchanged. Transcripts for PDGF-B and ET were increased in the early stages but declined to less than controls in the latter stages of MCT-induced hypertension. In contrast, levels of VEGF mRNA decreased to less than controls as the disease progressed. Viewed collectively, the diverse pattern of expression suggests that alterations in the levels of the growth factor transcripts may have a significant role in the development of pulmonary hypertensive disease and may be relevant to the pathological and structural changes in MCT-induced pulmonary hypertension.

A single subcutaneous injection of monocrotaline (MCT)*, a pyrollizidine alkaloid, into rats causes progressive lung injury accompanied by remodeling of the pulmonary vasculature, sustained pulmonary hypertension, and persistent defects in pulmonary ventilation [1–4]. Intercellular signaling mechanisms driving lung cell responses to MCT are not well understood. In this context, epidermal growth factor can be localized immunocytochemically around restructuring pulmonary vessels and airways after MCT administration [5]. Interleukin-1, a proinflammatory cytokine and vascular smooth muscle mitogen, can be detected by bioassay in bronchoalveolar lavage fluid from MCT-treated rats [6]. Preliminary experiments suggest that alveolar macrophages elaborate increased amounts of transforming growth factor-beta (TGF-β) early after MCT administration [7]. Collectively, these findings support the concept that multiple growth factors contribute to regulation of cellular responses in this model of lung injury and pulmonary hypertension.

In the present report we have examined changes in the abundance of selected growth factor mRNA transcripts in the lungs of rats as a function of time after MCT administration. A number of growth factor transcripts were studied, including TGF-β, platelet-derived growth factor (PDGF)-A and -B, transforming growth factor-α (TGF-α), endothelin (ET) and vascular endothelial cell growth factor (VEGF). As discussed subsequently, we focused on these mRNAs because of observations that the mature peptides evoke responses in cultured cells that can reasonably be incriminated in MCT-induced lung disease. We reasoned that information regarding changes in lung tissue mRNA for growth factors would be critical in designing future experiments to delineate cellular sources, target cells and pathogenic roles for specific growth factors in MCT-induced lung disease and related disorders.

* Abbreviations: MCT, monocrotaline; TGF, transforming growth factor; PDGF, platelet-derived growth factor; ET, endothelin; VEGF, vascular endothelial cell growth factor; Ppa, mean pulmonary artery pressure; RV, right ventricle; LV + S, left ventricle plus septum; RVH, right ventricular hypertrophy; ECM, extracellular matrix; and BM, basement membrane.

Materials and Methods

Animal model and physiologic assessment. Adult male Sprague–Dawley rats (Harlan Industries, Indianapolis, IN) weighing 200–225 g were given a subcutaneous injection of 60 mg/kg MCT (Transworld Chemicals, Inc., Rockville, MD) or an equivalent volume of its vehicle. Rats were subsequently killed at 1, 4, 7, 10, 14 and 21 days post-injection for the isolation of total RNA from lungs. A separate group of animals that were treated in an identical manner with either MCT or vehicle were anesthetized with 30 mg/kg sodium pentobarbital, and mean pulmonary artery pressure (Ppa) was measured. Then animals were killed by an overdose of sodium pentobarbital, and the heart and lungs were rapidly excised. The heart was dissected away from the lungs, and the right ventricle (RV) was separated from the left ventricle plus septum (LV + S) and weighed. The ratio of the weights of the RV to LV + S was determined as an index of right ventricular hypertrophy (RVH) [8].

Isolation of total RNA and northern analysis. Isolation of total RNA and northern analysis were performed as described previously [9] with minor modifications. Briefly, lungs from three control and three MCT-treated rats per time point were homogenized in a solution containing 4 M guanidinium isothiocyanate followed by purification of total RNA by cesium chloride density gradient centrifugation. The RNA pellet was suspended in diethylpyrocarbonate-treated water and quantitated spectrophotometrically at 260 nm. Equal amounts of the RNA samples (15 μg) were fractionated on a 1.2% agarose/2.2 M formaldehyde denaturing gel, transferred to a nylon membrane (Zeta Probe, Bio-Rad Laboratories, Richmond, CA) and hybridized with the appropriate radiolabeled cDNA probes. Large scale preparation of the DNAs was carried out after transformation of *Escherichia coli* strain HB101 with the plasmids and purified using the plasmid purification kit (Qiagen, Studio City, CA) as per the manufacturer's instructions. Isolation of the cDNA fragments from the purified plasmids using restriction enzymes and subsequent purification of the fragments from

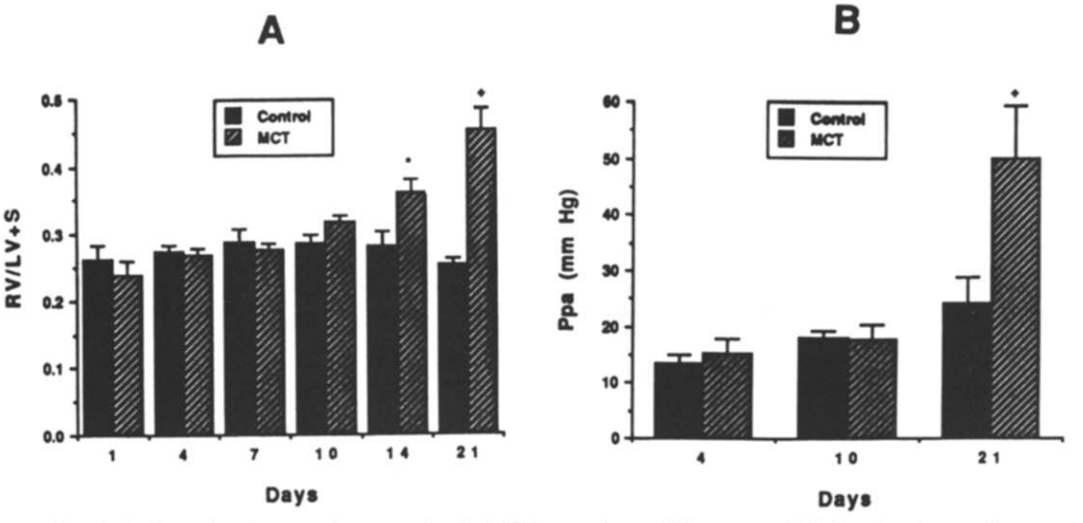

Fig. 1. Indices of pulmonary hypertension in MCT-treated rats. Histograms depicting the changes in right ventricular hypertrophy (RVH, panel A) and pulmonary arterial pressure (Ppa, panel B) as a function of time in MCT-treated rats compared with control rats. Values were determined from three control and three MCT-treated rats per time point and are means ± SEM. Symbols are used to denote those values that were significantly different from control: *P ≤ 0.05 and †P ≤ 0.01.

agarose gels were performed by standard protocols [10]. The cDNA fragments were labeled with [α-^{32}P]dCTP by the random-primed method using the ^{17}Quickprime kit (Pharmacia, Piscataway, NJ) as per the manufacturer's instructions. Hybridizations were carried out at 42° under stringent conditions in a solution containing 50% formamide for 12–16 hr followed by washes exactly as described by Claycomb and Lanson [9]. Autoradiography was performed by exposing the blots to X-ray film (Kodak X-Omat AR, Picker, Cleveland, OH) in the presence of an intensifying screen (DuPont Cronex lightning-plus, Picker, Cleveland, OH) at −70°. The intensity of the signals was quantitated by scanning densitometry, normalized to the 28S and 18S ribosomal RNA levels, and are reported as percent of controls (see Table 1). To examine the temporal changes in the growth factor transcripts from MCT-treated rat lungs, total RNA was isolated from control and experimental lung tissues and subjected to northern blot analysis. Lung tissues were pooled for RNA isolation from either three control rats or three MCT-treated rats per time point. In an attempt to verify the accuracy of the changes in the expression of individual growth factors with respect to each other, two separate blots were prepared for the northern analysis and the same two blots were stripped and reprobed sequentially with different probes.

Statistical analysis. Data for RVH and Ppa are expressed as the mean ± SEM. Single data point comparisons were analyzed by one-way analysis of variance. Statistical differences between groups were evaluated post-hoc by the Newman–Keuls test. A P value ≤ 0.05 denotes statistical significance [11].

Results

Indices of pulmonary hypertension in MCT-treated rats. To confirm the efficacy of MCT treatment, we measured Ppa and RVH as a function of time in control rats and rats given MCT. As shown in Fig. 1A, significant increases in RVH were observed by day 14 in MCT-treated rats compared with controls, which persisted for the duration of the study. Likewise, Ppa was elevated significantly by day 21 in the MCT-treated group (Fig. 1B).

Growth factor gene expression in MCT-treated rat lungs. As shown in Fig. 2, the abundance of TGF-β1 transcript was clearly increased in response to MCT from day 4 onwards (122% of control) and remained at elevated levels throughout the duration of the study with maximum increases on day 21 (165% of control). TGF-β2 and TGF-β3 transcripts showed increases over controls from day 4 onwards with maximal increases on day 10 for TGF-β2 (142% of control) and day 21 for TGF-β3 (161% of control) post-MCT treatment. TGF-α mRNA was found to be present at very low levels at all the time points except days 7 and 10, during which no convincing changes in response to MCT could be detected. Of the two PDGFs, the steady-state levels of mRNA for PDGF-A did not exhibit any changes in response to MCT, whereas those for PDGF-B showed increases on days 1 and 4 (132% of control) but was less than controls for the remainder of the study period (71 and 72% of control on days 14 and 21, respectively). Expression of ET mRNA was similar to that of PDGF-B with increases on days 1 and 4 (123 and 118% of controls, respectively) and maximal decrease on day 21 (66% of control). Expression of VEGF mRNA was less than controls at all time points with maximal decreases on days 7, 14 and 21 (40, 45 and 41% of controls, respectively). The results of the scanning densitometry for the various transcripts at all time points are shown in Table 1.

Discussion

This study examined growth factor gene expression in rat lungs as a function of time after administration of the pneumotoxin MCT. Our results clearly demonstrate that development of MCT-induced pulmonary hypertension is accompanied by temporal alterations in the expression of specific growth factor transcripts. The development of pulmonary hypertension was associated with early and sustained increases in the expression of TGF-β genes. Alterations in TGF-β have also been detected in other models of pulmonary disease. For example, bleomycin-induced pulmonary fibrosis is accompanied by increased lung TGF-β mRNA content [12] and by progressive appearance of immunoreactive TGF-β throughout the lung. TGF-β also has been detected in sheep lung lymph during evolution of air embolism-induced pulmonary hypertension

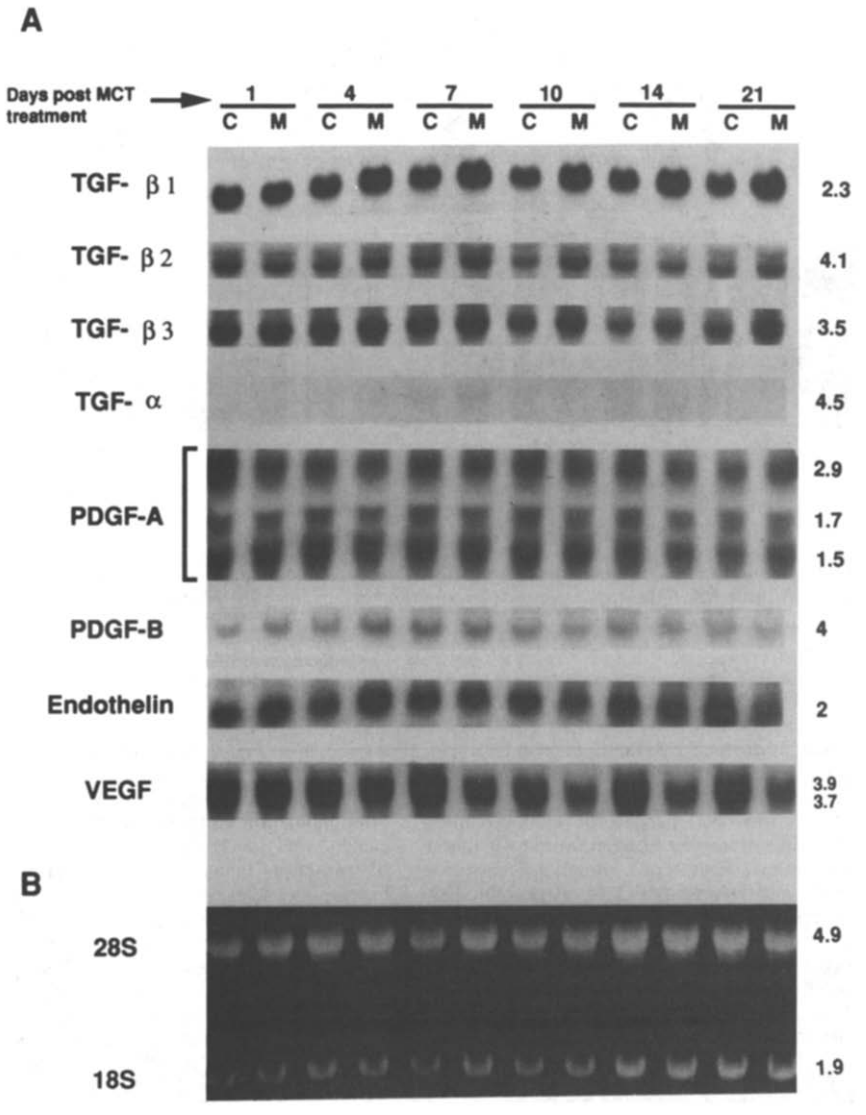

Fig. 2. Temporal alterations in the steady-state levels of growth factor transcripts in MCT-treated rats. (A) Northern analysis of total RNA isolated from pooled rat lungs (three per group) to determine changes in the steady-state levels of mRNAs for the various growth factors in MCT-treated rats (M) compared with control rats (C). The numbers on top indicate the days post-MCT treatment. The names of the various transcripts are shown on the left with their approximate sizes in kilobases shown on the right. (B) Picture of an ethidium bromide stained gel showing the relative amounts of RNA loaded on the gel.

[13]. Interestingly, unlike these other model systems, TGF-β1 is decreased in remodeling pulmonary arteries of newborn calves with hypoxic pulmonary hypertension as compared to normotensive arteries [14].

The TGF-β growth factors are multifunctional and their role appears to be fundamental in tissue injury and repair processes such as angiogenesis [15], chemotaxis of inflammatory cells and proliferation of fibroblasts [16]. Importantly, they have been demonstrated to play a pivotal role in enhancing the accumulation of extracellular matrix (ECM) proteins both at the level of synthesis of ECM proteins in wound repair and a number of pathologies [17]. Because MCT causes increased synthesis and deposition of ECM proteins in lung vasculature and parenchyma, and angiogenesis in the bronchial circulation [18], inflammatory cell infiltration into the lung [19], proliferation of fibroblasts and hypertrophy of vascular smooth muscle cells [20], it is reasonable to propose that TGF-β plays a central role in the pathogenesis of MCT-induced lung injury and pulmonary hypertension. Our present results provide the background for numerous additional experiments required to define the sources, cellular targets, and consequences of TGF-β accumulation in MCT-induced hypertensive pulmonary vascular disease.

The abundance of TGF-α transcripts did not change in response to MCT. A recent preliminary communication utilizing the polymerase chain reaction indicated that TGF-α mRNA can be detected in rat cultured pulmonary artery

Table 1. Scanning densitometric values for the alterations in growth factor mRNA levels (described in Fig. 2)

	Growth factor mRNA levels (% of control)					
	Days post-MCT treatment					
	1	4	7	10	14	21
TGF-β1	91	122	129	148	142	165
TGF-β2	96	113	106	142	86	113
TGF-β3	98	103	123	125	117	161
PDGF-A	98	82	93	88	85	111
PDGF-B	115	132	90	84	71	72
ET	123	118	86	88	88	66
VEGF	96	87	40	64	45	41

smooth muscle cells [31]. Immunoreactive TGF-α has been detected in arterioles associated with vascular beds other than lungs [22]. Several explanations exist to explain the failure of TGF-α mRNA abundance to change in MCT-treated rat lungs. First, it is conceivable that this growth factor does not contribute to the pathogenesis of MCT-induced lung injury and pulmonary hypertension. Second, alterations in the levels of TGF-α mRNA may occur in a circumscribed population of lung cells that are below the limits of detection by northern analysis of RNA from whole lung. Finally, TGF-α could be mobilized from cell membranes by elastase, including neutrophil derived elastase [22]. Consequently, the distribution and/or availability of this growth factor could be increased in MCT-treated lungs by mechanisms unrelated to increased abundance of its mRNA.

PDGF is a ubiquitous growth factor synthesized by a variety of cell types including endothelial cells and vascular smooth muscle cells [23]. Along with being a potent mitogen, PDGF also has shown to be a vasoconstrictor for isolated rat aortic strips [24]. While the expression of PDGF-A did not show any changes in response to MCT, the abundance of PDGF-B mRNA was elevated on days 1 and 4 post-MCT treatment and declined to less than controls during the later time points. Roth and coworkers [25] showed that neutralizing anti-rat PDGF antibodies fails to block development of lung injury and pulmonary hypertension in rats treated with dehydromonocrotaline, thus suggesting that the growth factor did not play a causal role in this model. It is not known whether the decline in PDGF-B transcripts demonstrated in the present study can be incriminated in development of MCT-induced pneumotoxicity. ET is a potent vasoconstrictor [26] and mitogen for fibroblasts and smooth muscle cells [27, 28]. Time-dependent changes in the abundance of ET mRNA mimicked that of PDGF-B; levels increased early after MCT administrations and then decreased below controls during development of pulmonary hypertension. A recent study has demonstrated increases in both the expression and production of ET-1 in fawn-hooded rats, a strain which develops idiopathic pulmonary hypertension, but not in hypoxia-induced pulmonary hypertension [29]. Furthermore, the increased expression and production of ET in these animals occurred prior to the development of hypertension. Previous studies in isolated perfused lungs from MCT-treated rats showed an increased vascular responsiveness during the early time points [8]. The early increases in the expression of both PDGF-B and ET, both vasoactive agents, complement this observation.

In contrast to PDGF-B and ET, expression of VEGF was unchanged until day 4 but was less than control from day 7 onwards. VEGF is a potent mitogen and angiogenic agent for endothelial cells and is known to be sequestered by ECM components like heparan sulfate [30]. While the significance of the decrease in VEGF mRNA is unclear at the moment, levels of VEGF peptide may increase during the development of pulmonary hypertension. Based on the observation that components of the basement membrane (BM) undergo considerable structural reorganization as early as day 4 post-MCT treatment (unpublished data), it is reasonable to propose that sequestered VEGF peptide is released during this process. Although speculative, it is possible that increased levels of the VEGF peptide may down-regulate the expression of its mRNA. Further analysis of the VEGF peptide content during the development of hypertension in this model by immunohistochemical and western blot analysis would lend support to this hypothesis.

It is appropriate to consider the limitations of a study of this design. While it seems reasonable to propose that growth factors whose expression increases or decreases may play a contributory role in MCT-induced pneumotoxicity, it is presumptive to suggest that growth factors whose expression remains unchanged after MCT administration are not involved in the pathogenesis of lung injury and pulmonary hypertension in this model. There are at least two reasons to emphasize this limitation. First, we assessed growth factor mRNAs in the whole lung; the method of northern analysis may not be sufficiently sensitive to detect changes in transcripts if they are confined to a limited cell population. Second, some of the growth factors examined may be regulated, at least in part, by mechanisms unrelated to changes in steady-state levels of mRNA. These other mechanisms may act to mobilize growth factors without commensurate changes in the abundance of their mRNAs. Although the absolute changes in the levels of the various growth factor transcripts in whole lung in response to MCT were small, it is important to re-emphasize the fact that differential changes within specific lung cell types may occur. Resolution of this possibility necessitates an additional series of studies utilizing *in situ* hybridization to determine the alterations of specific transcripts at a cellular level.

Structural alterations comprising both cellular and ECM proteins within the lungs are associated with the pathophysiological changes in MCT-induced hypertension. Biochemical and ultrastructural studies of MCT-treated rat lungs have provided evidence previously for an increased accumulation of elastin and collagen in the pulmonary artery walls concomitant with the development of pulmonary hypertension [31]. Studies utilizing immunohistochemical techniques on paraffin embedded sections of rat lungs have also demonstrated increases in the deposition of the BM-ECM components (fibronectin, laminin, type IV collagen and perlecan) in response to MCT prior to the development of pulmonary hypertension. These changes in BM components could be attributed, at least in part, to the increases in the steady-state levels of the mRNAs encoding these proteins (unpublished data). While these observations provide indirect evidence for the link between alterations in the structural components and the development of pulmonary hypertension induced by MCT, the roles of several growth and differentiation factors in orchestrating these changes are not clear. A vast majority of growth factors serve to alter the expression of several genes including the ones encoding structural proteins at the level of transcription. Thus, it is reasonable to propose that the increased expression of the genes encoding the structural proteins in this model of hypertension could be a consequence of the alterations in the expression and synthesis of specific growth factors. Although not an exhaustive survey, this analysis was designed as a first step in defining the role of growth factors in the development of MCT-induced pulmonary hypertension and warrants future studies designed to provide detailed analyses of the role of each growth factor.

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