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SHORT COMMUNICATION

Cellular Fibronectin and von Willebrand Factor Concentrations in Plasma of Rats Treated with Monocrotaline Pyrrole

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ABSTRACT. The monocrotaline pyrrole (MCTP)-treated rat is a useful model for the study of certain chronic pulmonary vascular diseases. A single, i.v. administration of a low dose of MCTP causes pneumotoxicity, pulmonary vascular remodeling, sustained increases in pulmonary arterial pressure, and right ventricular hypertrophy in rats. The pulmonary vascular lesions are characterized by endothelial cell alterations, platelet and fibrin microvascular thrombosis, pulmonary edema, and thickening of the intimal and medial layers of the vessel wall. These lesions suggest that some dysfunction of the hemostatic system occurs in the lungs of rats treated with MCTP. We evaluated the concentrations of two adhesion proteins, cellular fibronectin (cFn) and von Willebrand factor (vWF), in the plasma of rats treated with MCTP. We hypothesized that changes in these factors occur along with markers of pneumotoxicity and ventricular hypertrophy and that such changes might contribute to the genesis of the vascular lesions. Enzyme-linked immunosorbent assays were used to measure cFn and vWF concentrations in the plasma of rats after MCTP treatment. Rats treated with a single, i.v. injection of 3.5 mg MCTP/kg body weight had delayed and progressive lung injury characterized at 5 days post-treatment by increases in the lung-to-body weight ratio and in lactate dehydrogenase activity and protein concentration in cell-free bronchoalveolar lavage fluid (BALF). Values for these markers were further increased at 8 days and reached a plateau thereafter. The number of nucleated cells within the BALF was increased at 8 and 14 days. Right ventricular hypertrophy, an indirect marker of pulmonary hypertension, was evident at 14 days. The cFn concentration was increased in plasma of rats at 8 and 14 days after treatment with MCTP. There was no difference between the vWF concentration in plasma of rats treated with MCTP and those treated with vehicle at any time. We conclude that an increase in plasma cFn concentration occurs prior to the onset of right ventricular hypertrophy and that this change is consistent with a role for cFn in the genesis of vascular remodeling and pulmonary hypertension in the MCTP-treated rat. The lung vascular injury and pulmonary hypertension in this model were not reflected in altered vWF concentration in the plasma. BIOCHEM PHARMACOL 51;2:187–191, 1996.

KEY WORDS. lung injury; endothelial cell dysfunction; monocrotaline pyrrole; pulmonary hypertension; ventricular hypertrophy; von Willebrand factor; cellular fibronectin; vascular remodeling

A single, i.v. injection of a low dose of chemically synthesized MCTP¶ causes in the lungs of rats histologic lesions and biochemical alterations that are similar to those of people affected with certain chronic pulmonary vascular diseases, such as primary pulmonary hypertension and the late or proliferative phase of the adult respiratory distress syndrome [1, 2]. A characteristic lesion of MCTP toxicosis in rats is thickening of pulmonary vascular walls. Alterations observed commonly in the intimal layer include endothelial cell blebbing, swelling, and hypertrophy. Smooth muscle cell hypertrophy and hyperplasia occur in the medial layer of the vasculature [3].

The pulmonary vascular endothelium is a target for MCTP. Morphologic alterations in endothelial cells of the lung occur early during MCTP toxicosis *in vivo* [3], and barrier and metabolic functions of pulmonary endothelium are impaired [4–6]. MCTP administration to endothelial cell monolayers *in vitro* causes cell detachment, cytotoxicity, and inhibition of cell replication [6]. cFn is synthesized by several types of cells, including endothelial cells, and is found in the extracellular matrix of blood vessels. It binds to endothelial cells and to the substrata and is intricately involved in cell motility, morphol-

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¶ Abbreviations: MCTP, monocrotaline pyrrole; cFn, cellular fibronectin; vWF, von Willebrand factor; DMF, *N,N*-dimethylformamide; LW/BW, wet lung-to-body weight ratio; BALF, bronchoalveolar lavage fluid; LDH, lactate dehydrogenase; and RV/(LV+S), the weight of the right cardiac ventricle divided by the sum of the weights of the left ventricular wall plus interventricular septum.

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ogy, and adhesion [7]. It is concentrated in endothelial cells of blood vessels and to a lesser extent in the alpha granules of platelets [7, 8]. Release of increased amounts of cFn into the circulation has been associated with vascular injury in animal models of pulmonary vascular disease *in vitro* and *in vivo* [9, 10] and in people with vascular diseases [8, 11]. cFn deposition by injured cells provides a matrix for vascular repair processes such as angiogenesis [12]. Lipke and colleagues [13], using Northern analysis, demonstrated elevated levels of fibronectin mRNA in the lungs of rats treated with the parent compound, monocrotaline, 4 days previously. Increases in immunolocalizable fibronectin were also detected in the pulmonary vasculature 4 days after treatment with the toxicant compared with controls. We hypothesized that rats treated with MCTP would have an increased concentration of cFn in plasma.

In addition to endothelium, platelets are involved in the pathogenesis of MCTP-induced pulmonary hypertension. Platelets are sequestered in the lungs after rats are treated with MCTP [14]. Histologic examinations reveal platelet thromboembolic lesions that occur in the lungs of rats relatively soon after they are treated with MCTP [15]. Moreover, rats made moderately thrombocytopenic by administration of an antiplatelet serum are protected from the pulmonary hypertension that occurs after MCTP administration [16]. These results suggest that platelets may help to mediate the pulmonary vascular response to treatment with these toxicants. vWF is an adhesion molecule produced primarily by endothelial cells and to a lesser extent by megakaryocytes [17]. Since the endothelium of the lungs is altered and pulmonary platelet sequestration occurs in MCTP-treated rats, it seemed possible that MCTP treatment might alter production of vWF and that resultant increases in vWF concentration might be detected in plasma.

MATERIALS AND METHODS

Male, Sprague-Dawley rats (CD-Crl:CD*(SD)BR VAF/Plus*) (Charles River Laboratories, Portage, MI), weighing 200–250 g, were housed three per plastic cage on aspen chip bedding (Northeastern Products Corp., Warrensburg, NY) under conditions of high efficiency particulate filtered air and controlled temperature (18–21°), humidity (40–70%), and light cycle (light:dark 12:12). Rats were allowed food (Wayne Lab-Blox, Allied Mills, Chicago, IL) and tap water *ad lib*.

MCTP was synthesized from monocrotaline (Trans World Chemicals, Washington, DC) via an *N*-oxide intermediate as described by Mattocks [18], dissolved in the vehicle, DMF, and stored in the dark under nitrogen at 0°. Rats were given a single injection of either MCTP (3.5 mg/kg) or an equal volume of DMF vehicle (0.5 mL/kg) in a tail vein and were evaluated at 1, 5, 8, or 14 days after treatment. Each rat was anesthetized with pentobarbital sodium (50 mg/kg, i.p.), and the trachea was cannulated. Blood was drawn from the abdominal vena cava into a syringe containing 3.8% sodium citrate (9:1, blood:sodium citrate) and was dispensed into plastic tubes seated in an ice bath. Blood samples were spun in a centrifuge at 600 *g* and 4° for 20 min. Plasma was removed, and aliquots were frozen at −20° for later analysis.

After phlebotomy, each rat was exsanguinated. The trachea, lungs, and heart were excised *en bloc* and rinsed with saline. The lungs were lavaged twice with a predetermined volume of isotonic saline as described previously [19], and lavage aliquots were combined. Nucleated cells were enumerated in BALF using a hemocytometer. Cell-free supernatant fluid was prepared by spinning BALF samples in a centrifuge at 600 *g* for 10 min. Protein concentration and LDH activity were determined by the methods of Lowry *et al.* [20] and Bergmeyer and Bernt [21], respectively.

Right ventricular hypertrophy was assessed by comparing the ratio of right cardiac ventricular weight to the combined weights of the septum and left cardiac ventricular wall [22].

cFn in samples of rat plasma was determined by an enzyme-linked immunosorbent assay. Microtiter plates (Greiner; Alphen aan den Rijn, Netherlands) were coated overnight at 20° with mouse ascites fluid containing a monoclonal antibody (IgM) against the human cFn, which cross-reacts with rat cFn (Sigma Chemical Co., St. Louis, MO) (diluted 1:10 in PBS). After three washes with PBS containing 0.05% (v/v) Tween 20 (PBS/Tween), the wells were incubated with sample [diluted in PBS containing 2% (w/v) BSA (Sigma Chemical Co.: PBS/BSA] for 2 hr at 37°. Then, wells were washed three times with PBS/Tween, incubated for 2 hr at 37° with polyclonal, peroxidase-conjugated, rabbit-antiplasma fibronectin IgG (Dako A/S, Copenhagen, Denmark) (diluted 1:500 in PBS/BSA), and washed with PBS/Tween. Finally, 3,3′,5,5′-tetramethylbenzidine/H_2O_2 was added to the wells and acidified, and the absorbance was read at 450 nm in a Multiskan spectrophotometer (Flow Laboratories Ltd., Ayrshire, U.K.). Data were presented as micrograms per milliliter, relative to a standard of purified cFn (UBI, Lake Placid, NY) (30–300 ng/mL) in PBS/BSA. The protein content of the cFn standard solution was determined by the bicinchoninic acid protein assay procedure, as prescribed by the manufacturer (Pierce, Rockford, IL). Samples were run in triplicate, and the results were reported as a mean value. The assay was checked for specificity as follows. There was no reaction with fibronectin-depleted plasma (plasma absorbed on a gelatin-Sepharose column), commercially obtained, purified plasma fibronectin (UBI), or fibronectin from pooled plasma that had been absorbed and eluted from a gelatin-Sepharose column. Positive reactions were obtained with commercially obtained cFns (UBI and Sigma Chemical Co.), with conditioned medium from cultured human umbilical vein endothelial cells and rat endothelial cells, and with cFn purified from HT-1080 fibrosarcoma cells.

Samples of citrated rat plasma were analyzed for vWF antigen using the enzyme-linked immunosorbent assay of Tranquille and Emeis [23]. The plasma samples were assayed at a dilution of 1%. Anti-human vWF was obtained from Dakopatts (Glostrup, Denmark). Samples were run in triplicate, and the results were reported as a mean value. Data were expressed as percent relative to a pool of plasma from normal Wistar rats.

Data are expressed as means ± SEM. Homogeneity of variance was assessed with the F-max test. A log transformation was performed on nonhomogeneous data. If variances were

homogeneous, data were compared using an analysis of variance. Individual means were compared using the least significant difference test. Data with heterogeneous variances were analyzed with an adjusted Student's *t*-test for independent means [24]. The criterion for significance was $P < 0.05$.

RESULTS AND DISCUSSION

Several markers of pneumotoxicity were monitored to compare the development of lung injury with temporal changes in cFn (Fig. 1) and vWF (Table 1) concentrations. Rats treated with a single injection of MCTP had a moderate increase in the LW/BW at 5 days compared with controls, and a more marked increase thereafter. The LDH activity and protein concentration of cell-free BALF from MCTP-treated rats were moderately increased compared with control values at day 5. Increases in these markers were greater at day 8 and remained elevated at day 14. Rats that received MCTP had elevations in the number of nucleated cells in BALF at 8 and 14 days. Right ventricular hypertrophy, characterized by an increase in RV/(LV + S), was observed at day 14 in rats treated with MCTP. The plasma cFn concentration was increased significantly at days 8 and 14 after treatment with MCTP (Fig. 1). No treatment-related change in the vWF concentration in plasma of rats occurred at any time (Table 1).

In healthy people, cFn is localized to the endothelium of blood vessels [25]. cFn is synthesized and secreted into the medium by vascular cells in culture including endothelial cells, smooth muscle cells, and fibroblasts [26, 27]. Endothelial cells produce cFn in response to injury *in vitro* and *in vivo* [12, 28]. Therefore, increased concentrations of cFn in the circulation may serve as a useful marker of vascular injury [9].

Elevated concentrations of cFn have been detected in the plasma of human beings with collagen vascular diseases [11] and preeclampsia [8]. Measurement of cFn concentration has also proven useful in detecting experimental, acute pulmonary

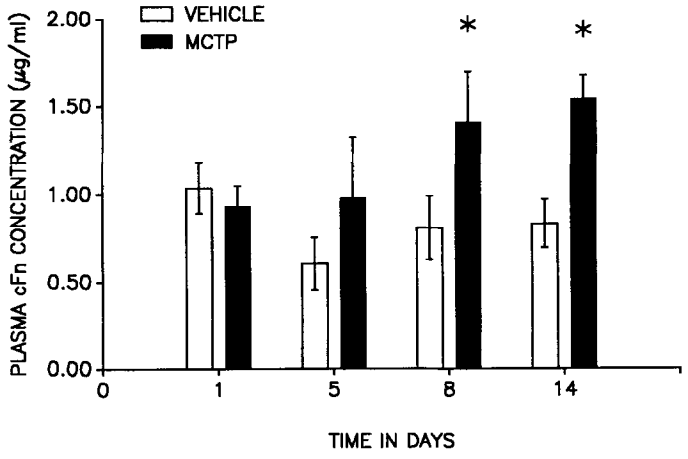

FIG. 1. Effect of MCTP on the concentration of cFn in plasma. Rats received a single i.v. injection of MCTP (3.5 mg/kg) or DMF vehicle at time 0. Values represent means ± SEM of 5–8 rats. Key: (*) significantly different from DMF control ($P < 0.05$).

vascular injury. An increased concentration of cFn was detected in the BALF and plasma of rabbits with acute pulmonary injury mediated by oxidants or leukocytes [10] and in BALF and perfusion medium of isolated rabbit lungs injured by O_2^- and H_2O_2 [9].

Our results indicate that the concentration of cFn in plasma of rats treated with MCTP 5 days previously tended to be greater than that of controls but was not statistically different. At 8 and 14 days after treatment of rats with MCTP, the cFn concentration in plasma was increased significantly. In correlative studies of the histologic lesions and biochemical changes that occur after rats are treated with MCTP, these times correspond with histologic evidence of vascular remodeling. At 5 days, minimal to mild thickening of some muscular and partially muscular arterial walls was detected. Morphometric analysis indicated that vessels with external diameter <60 µm were thickened. At 8 days after MCTP intoxication, morphometric alterations in pulmonary vessels were more severe, occurred more frequently, and extended to all levels of the pulmonary vasculature. Increases in pulmonary arterial pressure (days 8–14) and right ventricular hypertrophy (days 11–14) occur after treatment of rats with MCTP [3, 29]. Accordingly, the concentration of cFn in plasma may be a useful marker of the pulmonary endothelial cell injury and vascular remodeling in this animal model. The temporal relationship between increases in cFn concentration in plasma and alterations in the thickness of pulmonary vessel walls suggests the possibility that effects on cFn may give rise to some of the vascular changes, particularly those occurring later in the continuum such as smooth muscle hypertrophy and migration.

Extension of smooth muscle into small, normally nonmuscular arteries and morphologic alterations in vascular endothelial cells are characteristics of the lesions in lungs of MCTP-treated rats [3]. Boudreau *et al.* [30] demonstrated that increased fibronectin production by smooth muscle cells is associated with migration of smooth muscle and consequent intimal thickening in the ovine ductus arteriosus. Hinek *et al.* [31] postulated that increased fibronectin synthesis occurs as a result of release of elastin binding protein from smooth muscle cells and that this is critical for cell detachment and migration in this model. It was further determined that ductus arteriosus smooth muscle migration *in vitro* is related to increased production of fibronectin [32]. It seems feasible that MCTP-induced endothelial injury in rats resulted in increases in cFn that stimulated smooth muscle cell migration and thereby contributed to thickening of pulmonary vessels.

Rats treated with MCTP develop hypertrophy of the smooth muscle cells within the medial layer of pulmonary arteries. A progressive increase in fibronectin mRNA and increases in EIIIA and EIIIB exons have been associated with a rat model of cardiac hypertrophy [33]. In addition, fibronectin induces phenotypic alterations in cultured vascular smooth muscle cells that resemble those in lesions of hypertension [34]. Induction of fetal isoforms of fibronectin in smooth muscle cells has also been demonstrated as an adaptation to pressure overload in a rat model of cardiac hypertrophy [35]. Inasmuch as rats treated with MCTP develop pulmonary vascu-

TABLE 1. Markers of pneumotoxicity and ventricular hypertrophy

Days	Treatment	LW/BW (x 10^3)	BALF LDH activity (U/dL)	BALF Protein (μg/mL)	BALF TNCC (cells/μL)	RV/(LV + S)	Plasma vWF
1	Vehicle	5.06 ± 0.18	4.31 ± 0.23	130 ± 18	31 ± 6	0.29 ± 0.01	100 ± 14
	MCTP	4.76 ± 0.22	4.46 ± 0.31	145 ± 28	28 ± 1	0.30 ± 0.01	115 ± 13
5	Vehicle	4.55 ± 0.14	4.04 ± 0.25	91 ± 18	26 ± 4	0.30 ± 0.01	89 ± 10
	MCTP	5.86 ± 0.30*	12.29 ± 2.91*	558 ± 170*	43 ± 9	0.29 ± 0.01	104 ± 6
8	Vehicle	4.07 ± 0.10	4.29 ± 0.43	115 ± 4	26 ± 2	0.31 ± 0.01	91 ± 18
	MCTP	7.50 ± 1.02*	26.82 ± 5.63*	1452 ± 392*	82 ± 12*	0.32 ± 0.01	98 ± 11
14	Vehicle	3.89 ± 0.06	3.65 ± 0.21	104 ± 12	23 ± 2	0.29 ± 0.01	79 ± 17
	MCTP	7.59 ± 1.40*	13.14 ± 2.71*	950 ± 303*	86 ± 20*	0.48 ± 0.04*	104 ± 15

Rats received a single i.v. injection of MCTP (3.5 mg/kg) or vehicle at time 0. Values represent means ± SEM of 5–8 rats. Abbreviations: MCTP, monocrotaline pyrrole; LW/BW, wet lung-to-body weight ratio; BALF, bronchoalveolar lavage fluid; LDH, lactate dehydrogenase; TNCC, total nucleated cell count; RV/(LV + S), the weight of the right cardiac ventricle divided by the sum of the weights of the left ventricular wall plus interventricular septum; and vWF = von Willebrand factor.

* Significantly different from vehicle control ($P < 0.05$).

lar remodeling and sustained increases in pulmonary arterial pressure, it is tempting to speculate that the sustained alterations in the pulmonary vascular pressure partially contribute to the increased production in cFn in this model and that synthesis of the fetal forms of fibronectin occurs as endothelial and smooth muscle cells respond to injury.

Increased concentrations of plasma vWF have been associated with human conditions involving endothelial cell perturbation. It was suggested that people with elevated plasma vWF concentration are at increased risk for thrombosis [17]. Greater platelet adhesion to the subendothelium has been observed *in vitro* when the vWF concentration is increased in the subendothelium or in the plasma [36].

Agents such as histamine, thrombin, fibrin and endotoxin disrupt endothelial cell junctions and increase release of vWF from cell cultures *in vitro* [37, 38]. Phorbol myristate acetate and calcium ionophore, secretagogues that do not disrupt endothelial cell monolayer integrity, cause increased basolateral release of vWF from Weibel-Palade bodies [37]. In isolated, buffer-perfused rat lungs, acute lung injury induced by administration of phospholipase C and hydrogen peroxide causes increased release of vWF and the eicosanoids, thromboxane B_2 and 6-keto-$PGF1_\alpha$ [39]. The investigators concluded that release of vWF was a nonspecific marker of lung injury.

An increase in concentration of vWF antigen was identified in the plasma of patients with pulmonary hypertension that occurred secondary to cardiac dysfunction [40]. In this study, we sought to determine whether an increase in vWF concentration in plasma occurs in a rat model of chronic pulmonary vascular injury and pulmonary hypertension. No significant changes in the concentration of vWF in plasma occurred in MCTP-treated animals. The reason for the lack of increase in the face of obvious lung injury is unknown. It is possible that MCTP causes alterations in the pulmonary endothelium that are too modest to result in detectable changes in vWF in the systemic circulation. We cannot rule out the possibility that MCTP might have increased basolateral secretion of vWF or that dilution of pulmonary microvascular vWF in the systemic circulation might have precluded detection of changes.

In summary, we investigated the plasma cFn and vWF concentrations in a rat model of progressive lung vascular injury and pulmonary hypertension. The cFn concentration in plasma was increased significantly before the onset of pulmonary hypertension. Although more studies will be necessary for complete elucidation, the increase in this adhesion protein may help to explain a mechanism for cell migration, vascular thickening, and pulmonary hypertension in this model. In contrast to animal models of acute lung injury and a clinical report of pulmonary hypertension in people, we observed no change in vWF concentration in the plasma of rats intoxicated with MCTP.

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