Epidermal Growth Factor Receptor Blockade Mediates Smooth Muscle Cell Apoptosis and Improves Survival in Rats With Pulmonary Hypertension

Sandra L. Merklinger, PhD; Peter L. Jones, PhD; Eliana C. Martinez, MD; Marlene Rabinovitch, MD

Background—We previously reported that administration of elastase inhibitors reverses fatal pulmonary arterial hypertension (PAH) in rats by inducing smooth muscle cell (SMC) apoptosis. We showed in pulmonary artery (PA) organ culture that the mechanism by which elastase inhibitors induce SMC apoptosis involves repression of matrix metalloproteinase (MMP) activity and subsequent signaling through $\alpha_\beta_3$-integrins and epidermal growth factor receptors (EGFRs). This suggests that blockade of these downstream effectors may also induce regression of PAH.

Methods and Results—In this study, we first showed in PA organ culture that MMP inhibition or $\alpha_\beta_3$-integrin blockade with agents in clinical and preclinical use (SC-080 and cilengitide, respectively) mediates SMC apoptosis and regression of medial hypertrophy. We also documented similar results with an EGFR tyrosine kinase inhibitor. We then induced PAH in rats by injection of monocrotaline and, at day 21, began a 2-week treatment with SC-080, cilengitide, or the EGFR inhibitor PKI166. No vehicle- or cilengitide-treated animal survived beyond 2 weeks. Administration of SC-080 resulted in 44% survival at 2 weeks, and PKI166 therapy resulted in 78% and 54% survival in daily or 3-times-weekly treated animals, respectively. Four weeks after cessation of PKI166, we documented survivals of 50% and 23% in the 2 treatment groups, associated with reductions in pulmonary pressure, right ventricular hypertrophy, and abnormally muscularized distal arteries.

Conclusion—We propose that selective blockade of EGFR signaling may be a novel strategy to reverse progressive, fatal PAH. (Circulation. 2005;112:423-431.)

Key Words: cells ■ pulmonary heart disease ■ integrins ■ metalloproteinases ■ hypertension, pulmonary
sion of pulmonary vascular disease and complete reversal of fatal monocrotaline-induced PAH in rats by administering inhibitors of serine elastase activity. However, the lack of elastase inhibitors available for clinical use led us to test the efficacy of alternate apoptosis-inducing agents—an MMP inhibitor, an αβ3-integrin blocker, and an EGFR tyrosine kinase inhibitor (TKI)—in reversing experimentally induced pulmonary vascular disease in rats.

In the present study, we confirm regression of hypertrophy in rat PA organ cultures with agents available for clinical use that were designed to block MMP activity (SC-080) or αβ3-integrins (cilengitide). We also show induction of apoptosis of PA SMCs in cell as well as organ cultures by an EGFR TKI. We then induced PAH by injection of monocrotaline in rats and 21 days later, began a 2-week treatment with continuous intravenous cilengitide to block αβ3-integrins or with gavage administration of either the MMP inhibitor SC-080 or the EGFR blocker PKI166. No vehicle- or cilengitide-treated animals survived the 2-week treatment period. However, survival was 44% with SC-080 and 78% with daily EGFR TKI. Reversal of progressive disease in the EGFR TKI–administered animals was evident by decreased PAP, right ventricular hypertrophy (RVH), muscularized peripheral arteries, and recovery of a normal number of these vessels even 4 weeks after cessation of treatment.

Methods

Preparation of PA Organ Culture Explants

PAs were harvested from male Sprague-Dawley rats (225 to 250 g; Charles River Laboratories, Wilmington, Mass) 21 days after subcutaneous injection of monocrotaline (60 mg/kg) (Sigma) or saline (controls). The rats were euthanized by a lethal intraperitoneal injection of sodium pentobarbital (100 mg/kg), and the central PAs were harvested and incised longitudinally to expose the endothelial surface. Each explant was cultured in a 35-mm-diameter tissue-culture dish and embedded in a native type I collagen gel prepared as described previously. After hemodynamic assessment, all rats were monitored for an additional 4 weeks before measurement of PAP Measurements.

MMP, αβ3-Integrin, and EGFR TKI in Rats

Male Sprague-Dawley rats (225 to 250 g) were randomly assigned to different experimental groups 21 days after subcutaneous injection of saline (control animals) or 60 mg/kg monocrotaline to induce PAH. For αβ3-integrin blockade, rats received continuous intravenous cilengitide (15 mg/kg daily) or its vehicle (0.9% saline) via Alzet miniosmotic infusion pumps (Durect Corp). For MMP inhibitor studies, animals were gavage-fed daily doses of the MMP inhibitor SC-080 (25 mg/kg daily) in 0.5% methylcellulose and 0.1% Tween-80, or vehicle alone. For EGFR blocker studies, animals were gavage-fed once-daily or thrice-weekly gavage tube feedings of the EGFR-specific TKI, PKI166 (50 mg/kg; kindly supplied by Dr Peter Traxler, Novartis Pharma AG, Basel, Switzerland), or its vehicle (DMSO/0.5% Tween-80 diluted 1:20 [vol/vol] in water) was administered. A group of untreated rats was used for comparison in each inhibitor study. Treatment duration was 2 weeks in all instances. After EGFR TKI, the surviving rats were monitored for an additional 4 weeks before measurement of PAP and RVH. No detectable untoward effects of the inhibitors or their vehicles were noted in any of the saline-injected rats. Animals were euthanized early if they exhibited anorexia (≥10% weight loss over 2 consecutive days) or respiratory distress, in keeping with a protocol approved by the Animal Care Committee at Stanford University and following the guidelines of the American Physiologic Society.

PAP Measurements

PA catheterization was performed in rats anesthetized with 1% to 2% inhaled isoflurane in O2 (1 L/min) with a closed-chest technique as previously described. After hemodynamic assessment, all rats were euthanized with an overdose of anesthetic, the heart and lungs were removed en bloc, barium gelatin injections were performed, and the specimens were fixed by perfusion with 10% formalin.
SMC Apoptosis and Vascular Remodeling in PA Organ Cultures

<table>
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MCT indicates monocrotaline.

*P<0.0001, †P<0.01, ‡P<0.05.

Right Ventricular Hypertrophy

For assessment of RVH, the RV free wall was dissected from the left ventricle and septum (LV+septum) and weighed separately on an analytical scale. RVH was assessed by comparing RV/body weight and RV/(LV+septum).

Morphometric Analysis of Arteries in Rat Lung Tissue Sections

Transverse sections of the left lung were stained with hematoxylin and eosin as well as Movat’s pentachrome. In each lung section, the fully and partially muscularized and nonmuscular alveolar wall and duct arteries were counted in 5 to 10 randomly chosen fields at ×20 magnification, and each was expressed as a percentage of the total number of arteries at these levels. The total number of peripheral arteries was then calculated as a ratio of the number of arteries per 100 alveoli in each field. Morphometric analysis was performed by one observer blinded to the group from which the sections were taken.

Statistical Analysis

Data from multiple experiments are expressed as mean±SEM. Statistical significance was determined by 1-way ANOVA followed by Fisher’s least-significant-difference test for multiple comparisons. A probability value of <0.05 was considered significant. The number of animals analyzed in each group is indicated in the figure legends.

Results

MMP and α,β3-Integrin Inhibition

Similar to previous studies,18 we harvested rat PAs 21 days after injection of monocrotaline or saline and after 8 days of PA organ culture and quantified the number of apoptotic cells and their tissue distribution as judged by in situ TUNEL assays. Compared with vehicle-treated organ cultures, incubation with the MMP inhibitor SC-080 produced a 9-fold increase in TUNEL-positive SMCs, associated with a reduction in medial wall thickness and loss of elastic laminae (P<0.0001 for all) to values within the control saline-injected range (Table). Similarly, blockade with cilengitide, an antagonist selective for α,β3-integrins, brought about a 3-fold increase in TUNEL-positive SMCs (P<0.01), reflected by a reduction to control levels of both medial wall thickness and number of elastic laminae (P<0.0001) and number of elastic laminae (P<0.05) when compared with vehicle-treated PAs (Table).

Figure 1. Gelatin zymography of MMP-2 in lungs of rats injected with saline or monocrotaline. A, MMP-2 activity after saline and 2 to 21 days after monocrotaline injection. Upper band denotes pro form of MMP-2, and lower band at 62 kDa, active form. B, MMP-2 activity 21 days after monocrotaline or saline injection in rats treated once daily for 2 days with 25 to 100 mg of MMP inhibitor SC-080 or its vehicle. Densitometric analysis of active MMP-2 is shown in lower panel of each figure, but increase in pro form was seen at day 21 as well. Bars represent mean±SEM, n=4. *P<0.02 compared with saline-injected animals; †P<0.03 compared with vehicle-treated rats. Rel. indicates relative.

MMP and α,β3-Integrin Inhibition in Rats With PAH

We therefore pursued the hypothesis that SC-080 and cilengitide would, in a manner previously shown with elastase inhibitors,17 induce reversal of fatal PAH in rats. To this end, we applied gelatin zymography to lung tissue extracts and demonstrated a net increase in MMP-2 activity related to both the pro and active forms that was significant 21 days after monocrotaline injection (P<0.02; Figure 1A). A once-daily dose for 2 days of SC-080 was then given by gavage to rats beginning 21 days after monocrotaline, and MMP-2 activity in lung extracts was reduced to within the control range with a dose of SC-080 as low as 25 mg (P<0.03; Figure 1B). Male Sprague-Dawley rats were then injected with either saline or monocrotaline and 21 days later were administered the MMP inhibitor SC-080 or vehicle by daily gavage. A similar study
was performed with the αβ3-integrin blocker cilengitide or its vehicle by continuous intravenous infusion with a miniosmotic pump, as described in Methods. Despite confirmation of circulating plasma levels of cilengitide similar to those documented when the agent was administered to block angiogenesis in tumors (performed at Merck, Darmstadt, Germany), survival curves in cilengitide-treated rats were similar to those of vehicle controls (Figure 2A), as was the severity of RVH (Figure 2B). Despite subsystemic levels of mean PAP, we noted hepatomegaly, edema, and weight loss in rats that died after injection of monocrotaline, consistent with congestive heart failure.

Administration of the MMP inhibitor SC-080, however, resulted in 44% survival at 14 days compared with no survival in vehicle-treated rats by 12 days after initiation of treatment (Figure 2C). Compared with saline controls, there was a 2-fold increase in mean PAP (P<0.0001), from ≈18±0.6 mm Hg to ≈40±1.4 mm Hg in vehicle-treated rats, as assessed when the severity of weight loss or respiratory distress necessitated their euthanasia, as described in Methods. Mean PAP values in rats surviving 14 days after SC-080 treatment were intermediate and not significantly different from either saline-injected control or vehicle-treated, monocrotaline-injected rats (Figure 2D); RVH was, however, similarly increased in the SC-080– and vehicle-treated, monocrotaline-injected rats relative to saline-injected controls (Figure 2E).

**EGFR TKI in Cell and PA Organ Cultures**

We next determined whether direct blockade of EGFR signaling could, through loss of survival signals, induce SMC apoptosis in cell and PA organ cultures. Primary rat PA SMCs were seeded onto proteolyzed (heat-denatured) collagen. In contrast to the normal stellate appearance of cells grown on native type I collagen (Figure 3A), cells grown on proteolyzed collagen exhibited enhanced spreading (Figure 3B) in association with expression and secretion of TN-C (Figure 3C and 3D). The increased TN-C in the extracellular matrix cluster of αβ3-integrins, thereby activating EGFRs that promote SMC survival.14 This system of growing cells on proteolyzed collagen can then be used to test the efficacy of EGFR blockade in inducing SMC apoptosis. Compared with IgG-treated PA SMCs, blockade of EGFR signaling with the TKI AG1478 resulted in a 4-fold induction of apoptosis, as
Experiments were repeated 3 times in triplicate.

Figure 3. Blockade of α3β1-integrins and EGFRs in PA SMCs. Representative photomicrographs of primary PA SMCs grown for 24 hours on native type I (A) and proteolyzed (B) collagen. Immunodetection of TN-C under both cell-culture conditions (C and D). E, Percentage of SMC apoptosis by TUNEL assay 48 hours after αβ3-integrin blockade or EGFR inhibition, alone or in combination, and after control IgG incubation. Bars represent mean±SEM. Experiments were repeated 3 times in triplicate. *P<0.04 compared with IgG control cultures. Ab. indicates antibody.

judged by TUNEL-positive SMCs (P<0.04), similar to that seen with αβ3-integrin blockade. When both αβ3 integrin and EGFR signaling were blocked simultaneously, there was no significant additional apoptosis (Figure 3E).

We next determined whether the EGFR TKI AG1478 could induce regression of hypertrophied PA PAs in organ culture. A similar percentage of cells (10% to 14%) in PA organ cultures from saline- (Figure 4A) or monocrotaline-injected (Figure 4B) rats was TUNEL positive after DMSO-treatment after 8 days in culture. Four incremental doses of AG1478, from 250 nmol/L to 100 μmol/L, did not alter these values in PA organ cultures from saline-injected rats (not shown) but induced a >6-fold increase in TUNEL positivity in hypertrophied PA organ cultures from monocrotaline-injected rats, primarily within the vessel media but also in the adventitia and endothelium (Figure 4C–4F). The percentage of these cells was quantified and found to be similar for all TKI AG1478-treated cultures relative to vehicle and saline controls (P<0.0001; Figure 4G). We correlated the induction of SMC apoptosis with a dose-dependent regression in medial wall thickness (Figure 4H) and a reduction in the number of elastic laminae (Figure 4I) to values observed in saline control PAs (P<0.0001 for both).

EGFR TKI in Rats With PAH

We therefore determined whether a different EGFR TKI currently available for clinical use would reverse PAH when given to rats. As described earlier, rats were injected with monocrotaline or saline, and 21 days later, treatment was begun with daily or thrice-weekly gavage administration of the EGFR TKI, PKI166. At the end of a 2-week treatment regimen, any surviving animals were maintained under close observation for an additional 4 weeks without inhibitor therapy. Compared with no survival in the untreated or vehicle-treated rats, EGFR antagonism with PKI166 resulted in 78% and 54% survival for the daily and thrice-weekly treatment regimens, respectively (Figure 5A). At the end of a 4-week “recovery” period, there was a persistent 50% and 23% survival in these respective groups. Long-term survival was associated with signs of physical improvement, including weight gain, in all but 1 animal in these groups (data not shown). In contrast to a nearly 3-fold increase in mean PAP from 18±0.2 mm Hg measured in saline controls to 48±1.2 mm Hg in vehicle-treated rats before euthanasia, PAP values 4 weeks after cessation of treatment in PKI166-treated animals were only 31±5.3 mm Hg (P<0.01; Figure 5B) and were associated with a decrease in RVH (P<0.01; Figure 5C).

PKI166 administration was also associated with a decrease in muscularization of alveolar duct arteries relative to vehicle-treated rats (P<0.05) and a trend toward lower values of muscularized alveolar wall arteries (Figure 5D–5F). The loss of peripheral arteries assessed relative to alveoli observed in vehicle-treated rats was no longer apparent in those treated with PKI166 (P<0.05; Figure 5G–5I).

Discussion

In this study, we first established in PA organ culture with agents in clinical or preclinical use that inhibition of MMPs and αβ3-integrins and EGFR blockade induce regression of medial hypertrophy through SMC apoptosis and loss of elastic laminae. We then used these agents in the intact animal to reverse progressive PAH. The greatest efficacy appeared with daily EGFR TKI therapy, resulting in 78% survival at the end of the treatment period and 50% survival 4 weeks after cessation of therapy, in association with reduced PAP, RVH, and arterial remodeling.

Serine elastase activates MMPs both directly and indirectly by inactivating tissue inhibitors of MMPs and also increases MMP expression through the release of fibronectin peptides. Increased serine elastase activity is evident in the pulmonary circulation 2 days after injection of monocrotaline, but in the present study, although there was a trend toward an early increase by day 7, a significant elevation in lung MMP activity was not documented until 21 days after injection. This may be related to the difficulties inherent in quantitatively assessing zymographic data. Nonetheless, we were able to suppress MMP activity by giving SC-080. The decrease in the pro as well as the active form of MMP-2 also suggested that there was a positive-feedback mechanism that
enabled the inhibitor to limit expression and activity of the enzyme.

The role of MMPs in systemic vascular pathologies has been well documented. To this end, the broad-range MMP inhibitor Batimastat limited expansion of experimental abdominal aortic aneurysms in rats by attenuation of the inflammatory response, which was correlated with areas of elastin preservation. In the context of neointima formation induced by balloon-catheter injury in rat carotid arteries, MMP inhibition with Ilomastat (GM6001) resulted in a 97% decrease in the number of SMCs that migrated into the neointima. However, the beneficial effect of MMP inhibitors in limiting neointimal formation has been largely transient, and no studies have addressed their efficacy in reversing established vascular disease.

Our data showing a lack of efficacy of MMP inhibitors relative to previous results with elastase inhibitors suggest that factors induced by elastase but independent of MMP activation need to be suppressed. For example, it is possible that elastase-mediated release of growth factors in the intact rat is sufficient to prevent apoptosis. It is also possible that even with suppression of MMPs, there is enough matrix degradation to induce clustering of $\alpha_\beta_3$-integrins and mediation of cell survival through EGFRs.

Integrins, which mediate cell survival, proliferation, and migration, have been targeted to induce apoptosis of adherent cells in intact animals. Blockade of the pathological increase in $\alpha_\beta_3$-integrin expression in response to angiogenic growth factors and proteolyzed collagen will initiate both endothelial and SMC apoptosis. However, despite confirmation of this effect in our organ culture experiments, $\alpha_\beta_3$-integrin blockade in the intact animal failed to arrest progression of advanced PAH. This discrepancy could be due to activation of alternate matrix-ligand interactions that, as postulated earlier, could also induce survival signals from growth factor receptors. For example, in previous studies documenting the plasticity of the hypertrophied PA organ culture, repression of TN-C production led to upregulation of osteopontin, another $\alpha_\beta_3$-integrin ligand. It is also possible that the dose used in previous studies to inhibit tumor angiogenesis (data available through S. Goodman, Merck, Darmstadt, Germany) was insufficient to induce regression of monocrotaline-induced pulmonary vascular disease.

The potential SMC survival signal via EGFR led us to test the efficacy of TKI in our animal model. The mechanism of EGFR-mediated survival is related to tyrosine phosphorylation and activation of intracellular signal transduction pathways, such as phosphatidylinositol 3-kinase/AKT and the

**Figure 4.** Induction of apoptosis in PA organ cultures treated with EGFR blocker. Representative photomicrographs of TUNEL staining for nuclei from rat PAs 21 days after saline (A) or monocrotaline (B) injection. Positive TUNEL staining of SMCs after TKI of EGFR with AG1478 at 250 nmol/L (C), 10 μmol/L (D), 32 μmol/L (E), and 100 μmol/L (F) concentrations for 8 days. Quantification of TUNEL-positive cells (G), medial wall thickness (H), and number of elastic laminae (I). Data represent mean±SEM, n=4. *P<0.0001 compared with DMSO-treated PA organ cultures.
ras/raf/MEK/MAPK pathways. TKI inhibition works via competitive inhibition of the binding of ATP to the TK domain of the receptor, which results in attenuated autophosphorylation.

PKI166-induced SMC apoptosis has been correlated with downregulation of phosphatidylinositol 3/AKT signaling.

Targeting the EGFR pathway with antibody preparations inhibited SMC proliferation in balloon-injured rat carotid arteries and induced regression of human tumor xenografts in mice. The class of orally administered, low-molecular-weight compounds that block EGFR TK that we chose for our studies offers therapeutic advantages over receptor-specific antibodies, including drug availability, attenuated immunologic reactivity, direct intracellular effects on EGFR, and limited side effects.

In our experience, EGFR TKI with PKI166 resulted in improved long-term survival with associated reversal of progressive vascular remodeling. We speculate that EGFR blockade targeted apoptosis of SMCs that had abnormally proliferated. Although we did not induce complete reversal of structure and function to control levels, as with elastase inhibitors, the present study shows that survival was similar at the end of the treatment period and that PAP was decreased 1 month after cessation of therapy, in conjunction with evidence of physical improvement (eg, weight gain).

The mechanism accounting for the loss of peripheral pulmonary arteries after monocrotaline is endothelial and perhaps pericyte apoptosis. Despite the potential for EGFR blockade to induce endothelial cell apoptosis, we actually observed a return toward normal numbers of peripheral arteries. By reducing arterial muscularity and decreasing PAP, it is possible that new pulmonary vessel growth is stimulated. We do not anticipate a further drop in RV pressure or a regression of vascular disease during a longer period of therapy.

**Figure 5.** Survival, hemodynamic assessment, and morphometric analysis after EGFR blockade. A, Kaplan-Meier survival curve of monocrotaline-injected rats that received either no treatment (Tx), daily administration of vehicle, or daily or thrice-weekly administration of EGFR TKI, PKI166. Saline-injected rats receiving identical treatment were used as controls (data not shown). B, PAP measurements in saline-injected (n=12) and surviving monocrotaline-injected, PKI166-treated rats (n=7) 4 weeks after cessation of therapy. PAP in vehicle-treated MCT rats was made just before euthanasia (n=5). C, Ratio of RV to LV plus septum (S) weight as index of RVH in PKI166-treated animals. D and E, Representative light microscopic photographs of Movat pentachrome–stained lung tissue, showing monocrotaline-induced increase in arterial muscularization (E) compared with saline control (D). Arrows denote alveolar duct–associated arteries. F, Graphic representation of percentage of muscularized alveolar duct (Musc. AD) arteries. Representative light microscopic photograph of Movat pentachrome–stained lung tissue, showing monocrotaline-induced decrease in arterial number (G) compared with saline control (H). Arrows denote alveolar duct and wall arteries. I, Morphometric analysis of fully and partially muscularized alveolar duct and wall PAs relative to 100 alveoli. Bars represent mean±SEM. *P<0.01 compared with vehicle-treated rats. †P<0.05 compared with saline control rats.
follow-up, although this would be of interest. In previous studies, a diseased lung was removed 3 weeks after monocrotaline injection and was pressure-offloaded by transplanting it into a normal animal. This resulted in regression of vascular changes as judged by morphometric assessments 2 weeks later. There was, however, no evidence of ongoing apoptosis, suggesting that the changes had taken place very soon after pressure offloading.

The vascular changes that occur secondary to monocrotaline injection in the rat do not reflect the spectrum of occlusive and plexiform lesions seen in patients with primary or even advanced secondary pulmonary hypertension, so it will be interesting to determine, at least in organ cultures of tissue from lungs removed at transplantation, whether these lesions also regress with EGFR blockade. If so, this would support future consideration of the therapeutic potential of EGFR blockade for patients with PAH, as has been suggested for the treatment of a variety of cancers.

Acknowledgments

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References


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