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Effect of Maternal Administration of Betamethasone on Peripheral Arterial Development in Fetal Rabbit Lungs

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Key Words
Lung, fetal rabbit · Betamethasone, antenatal · Vascularization, lung · Alveolar epithelial cells, Flk-1-positive

Abstract
Objectives: Glucocorticoids promote lung maturation and reduce the incidence of respiratory distress syndrome in premature newborns. We hypothesized that betamethasone (BM), which is known to induce thinning of the alveolar walls, would also thin the arterial media and adventitia of intraparenchymatic vessels in developing rabbit lungs. ***Study Design:*** 112 fetuses from 21 time-mated, pregnant, giant white rabbits received maternal injections of BM at either 0.05 or 0.1 mg/kg/day on days 25–26 of gestational age. Controls received either saline (10 does, 56 fetuses) or no injection (10 does, 59 fetuses). Fetuses were harvested from day 27 onwards until term (day 31). 44 additional fetuses (8 does) were harvested between days 23 and 26. Endpoints were wet lung-to-body weight ratio, vascular morphometric indices and immunohistochemistry staining for α-smooth muscle actin, Flk-1, vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS). ANOVA (Tukey's test) and independent t test ($p < 0.05$) were used for comparison between BM and saline groups. ***Results:*** Maternal BM injected on days 25–26 to pregnant rabbits induced a significant decrease in fetal body and lung weight and the lung-to-body weight ratio in the preterm pups shortly after injection. BM led to a dose-dependent thinning of the arterial media and adventitia (pulmonary arteries with an external diameter (ED) of <100 μm), to an increase in the percentage of non-muscularized peripheral vessels (ED <60 μm), in eNOS and VEGF immunoreactivity of the endothelial and smooth muscle cells in the pulmonary vessels and to an increase in Flk-1-positive pulmonary epithelial cell density. ***Conclusions:*** Maternal administration of BM caused thinning of the arterial wall of pulmonary vessels (ED <100 μm) and a decrease in muscularization in peripheral vessels (ED <60 μm). This coincided with increased expression of Flk-1 in the endothelium and smooth muscle cells of the pulmonary arteries. All the effects studied were dose-dependent.

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Introduction

Preterm delivery occurs in 7–10% of all pregnancies and remains a major cause of infant mortality and morbidity mostly due to the consequences of respiratory distress syndrome and bronchopulmonary dysplasia. In neonatal pulmonary hypertension, the arteries fail to dilate normally due to the immature pulmonary arterial smooth muscle cells, which are supposed to remodel, relax and become thinner [1]. Over the years, several trials have shown the beneficial effects of antenatal administration of glucocorticoids (GC) by promoting lung maturation and reducing the incidence of respiratory distress syndrome in premature newborns [2]. GC increase surfactant pool size, decrease protein leak, increase air space and induce thinning of the alveolar walls, hence improving ventilatory measurements and gas exchange [3]. However adverse effects have been reported, such as a decrease in fetal body, lung and liver weights as well as brain development. Both effects seem to be dependent on the dose and timing of administration of steroids [4, 5].

We, as well as others, have used the rabbit as a model for fetal lung development in the study of different perinatal conditions [6, 7]. To a certain extent the lung development of rabbits mimics that of the human lung [8]. At term, rabbit lungs are in the terminal stage of saccular morphology with alveolization starting already prior to birth. Of interest to experimental medicine, rabbit does have a short gestational period, a large litter size and are inexpensive. Administration of betamethasone (BM) to pregnant rabbit does was previously shown to increase surfactant proteins A and B in the fetal lung, to improve ventilatory function and gas exchange in parallel with dose-dependent fetal growth restriction and decrease in fetal lung weight [3]. So far, the specific effects of antenatal GC on vascular morphology have not yet been studied.

Vascular endothelial growth factor (VEGF) is a potent vascular endothelial cell-specific mitogen, critical for developmental angio- and vasculogenesis, next to pneumocyte proliferation and type-II alveolar epithelial cell differentiation [9, 10]. It further stimulates microvascular permeability and vasodilatation. Most of the effects of VEGF are induced through its two tyrosine kinase receptors, VEGFR1 (Flt-1) and VEGFR2 (Flk-1/KDR) [11]. VEGF regulates endothelial nitric oxide synthase (eNOS) activity and expression [12] in endothelial cell cultures, an effect mediated by activation of the Flk-1/KDR receptor. Nitric oxide (NO) inhibits smooth muscle cell migration and activity of MMP-2, one of the zinc-dependent metalloproteases, which has been specifically implicated in smooth muscle cell migration. Dexamethasone was recently shown to inhibit in vitro production and activity of MMP-2 both directly as well as through enhancing NO production [13, 14].

We aimed to determine the morphologic effects of maternal administration of a single course of BM (canalicular phase) on pulmonary vascular development using a morphometric and immunohistochemical approach. We hypothesized that GC, causing thinning of the alveolar walls, would have similar effects on the arterial media and adventitia of intra-parenchymal vessels and promote the vascular transition to extra-uterine life.

Material and Methods

Rabbits were housed at 17 days of gestation in separate cages at normal room temperature and normal daylight, with free access to food and water. A total of 264 fetuses from 49 time-mated, pregnant, giant white rabbits were included in this experiment. Does and their fetuses were assigned to 2 BM (Celestone®, Schering-Plough, Brussels, Belgium) administration groups receiving either 0.05 mg/kg/day (BM = 0.05, 11 does, 62 fetuses) or 0.1 mg/kg/day (BM = 0.1, 10 does, 50 fetuses) injected on days 25 and 26, with a 24-hour interval between [3]. This corresponds to the canalicular stage of lung development. Control groups received either 2 injections of placebo (0.9% NaCl 0.2 ml/day, 10 does, 50 fetuses) or were not injected (10 does, 58 fetuses). We aimed to have at least 2 does for each different time point. The earlier stages of lung development were determined in 8 does, carrying 44 fetuses that were harvested on days 23–26. Animals were treated according to current guidelines on animal well-being and the Ethics Committee for Animal Experimentation of the Faculty of Medicine of the Katholieke Universiteit Leuven approved the experiments.

Sacrifice and Tissue Processing

Does were euthanized with an intravenous bolus (1 ml/kg) of a mixture of embutramide 200 mg, mebezonium 50 mg and tetracaine hydrochloride 5 mg (T61®; Hoechst Marion Roussel, Brussels, Belgium). Fetuses were delivered by cesarean section 20 min later to ensure that all fetuses were dead and no respiration of air would take place. Macerated fetuses were recorded as non-survivors and excluded from further analysis. During necropsy, the fetus was weighed using a scale measuring accurately up to 0.001 g (HF 2000; A&D Instruments, Haasrode, Belgium). The lungs were removed as a whole, separated from the trachea, and the right and left lungs were weighed. The right lung was snap frozen in liquid nitrogen and stored at –80°C for later biochemical and molecular studies, which are not the subject of this report. The left lung was immersed in 6% neutral buffered formalin solution for 24 h. After fixation, all left lungs were embedded in paraffin, cut into 5-μm mid-sagittal sections through the entire lung [15] for further histological and immunohistochemical staining. For vascular morphometry the slides were stained with Elastica van Gieson (Hart's method). The air space-tissue fraction was evaluated on hematoxyline-eosine-stained slides by the point-counting method and was used to correct the density of cells [16].

Table 1. Time course of changes in LBWR, and density of Flk-1-positive alveolar epithelial cells in fetal rabbit lungs after maternal administration of betamethasone (mean ± SEM)

	Day 23	Day 24	Day 25	Day 26	Day 27	Day 28	Day 29	Day 30	Day 31
Control	n = 8	n = 12	n = 12	n = 12	n = 13	n = 10	n = 11	n = 14	n = 11
LBWR	0.022 ± 0.001	0.027 ± 0.000	0.032 ± 0.001	0.030 ± 0.000	0.031 ± 0.001	0.028 ± 0.002	0.026 ± 0.001	0.027 ± 0.001	0.022 ± 0.000
Flk-1 epithelial	9,523 ± 600	10,372 ± 741	8,512 ± 554	6,892 ± 721	5,787 ± 412	5,900 ± 459	4,857 ± 358	4,512 ± 285	3,925 ± 311
Placebo					n = 6	n = 14	n = 11	n = 12	n = 13
LBWR					0.031 ± 0.001	0.029 ± 0.001	0.028 ± 0.001	0.022 ± 0.001	0.019 ± 0.001
Flk-1 epithelial					6,229 ± 586	6,085 ± 431	5,337 ± 345	4,956 ± 358	4,212 ± 314
BM = 0.05					n = 10	n = 12	n = 10	n = 13	n = 13
LBWR					0.026 ± 0.002	0.025 ± 0.001°	0.026 ± 0.001	0.020 ± 0.001	0.020 ± 0.001
Flk-1 epithelial					1,1530 ± 734°	5,500 ± 395	7,300 ± 678	6,432 ± 636	5.158 ± 427
BM = 0.1					n = 12	n = 10	n = 11	n = 6	n = 6
LBWR					0.025 ± 0.001*	0.027 ± 0.001	0.025 ± 0.001	0.022 ± 0.001	0.018 ± 0.000
Flk-1 epithelial					23,742 ± 742*	19,134 ± 785*	17,134 ± 654*	12,522 ± 732[a]	10,517 ± 643*

LBWR = Lung-to-body weight ratio; Flk-1 epithelial = density of Flk-1-positive parenchymal epithelial cells.
Differences between * BM = 0.1 and placebo and between ° BM = 0.05 and placebo are considered significant at $p < 0.05$.

Immunohistochemical Procedures

Briefly, after deparaffinization, endogenous peroxidase blocking (methanol with 0.3% H_2O_2; 30 min) and rinsing with phosphate-buffered saline (PBS; 0.01 M, pH 7.3), microwave heat retrieval was performed, followed by rinsing in PBS. For α-smooth muscle actin (α-SMA) no heat retrieval was required. After preincubation for 30 min with 10% normal goat serum (X0907 DakoCytomation, Glostrup, Denmark), the slides were incubated with mouse anti-human monoclonal anti-α-SMA (M0851, DakoCytomation; 1:100, 30 min), or with mouse anti-human monoclonal anti-eNOS (610296 Transduction Laboratories, Lexington, Ky., USA; 1:25, 2 h), or with mouse anti-human monoclonal anti-VEGF (MS-350-P1, NeoMarkers, Calif., USA; 1:50, 1 h), or with mouse anti-human monoclonal anti-Flk-1 (sc-6251, Santa Cruz Biotechnology, Calif., USA; 1:50, 30 min). After rinsing, the slides stained for α-SMA were incubated with goat anti-mouse biotin-labelled IgG (E0354, DakoCytomation; 1:400, 30 min), rinsed again and incubated with AB complex/HRP (K0355, DakoCytomation; 30 min). The slides stained for eNOS, VEGF and Flk-1 were incubated with peroxidase-conjugated EnVision™ + reagent (K4001, DakoCytomation; 30 min). Then all slides were rinsed with PBS, incubated with peroxidase substrate solution containing DAB (K3468, DakoCytomation, 10 min), rinsed with distilled water, counterstained with hematoxylin, dehydrated and mounted. Negative controls for specificity consisted of the omission of the primary antibody.

Gross Anatomy

Fetal body weight (FBW) and total wet fetal lung weight (TLW) enabled calculation of the lung-to-body weight ratio (LBWR).

Microscopic Parameters

In non-overlapping fields up to 50 peripheral vessels with an external diameter (ED) of ≤100 μm were measured, which correspond to the pre- and intra-acinar arteries in rabbit lungs and are believed to be the resistance arteries [17]. All diameters were measured at a magnification of ×200 along the shortest axis of the vessel [18] with a Zeiss Axioplan light microscope (Carl Zeiss, Oberkochen, Germany).

Vascular morphometric parameters include adventitial diameter (AD, μm); ED (μm); internal diameter (ID, μm). From these the following variables can be calculated: proportionate medial thickness (%MT = (ED – ID)/ED × 100) and proportionate adventitial thickness (%AT = (AD – ED)/ED × 100). The latter two parameters are proportionate values, nullifying the effects of vasodilatation, vasoconstriction and fixation on measurements as shown earlier by several authors both in human and animal specimens [18, 19, 36].

Muscularization of Pulmonary Vessels. Semiquantitative identification of the presence of smooth muscle bundles in the arterial and arteriolar wall was performed on slides stained for α-SMA. For all vessels with an ED of ≤100 μm, the diameter was noted and whether it was positively or negatively α-SMA stained. The vessels were empirically categorized into 3 classes (≤30, 30–60 and 60–100 μm) in order to assess the extent of peripheral muscularization along the acinar and pre-acinar vessels, as described previously [7]. The percentages of muscularized vessels in intra-acinar (<30 μm) vessels and beyond (30–60 and 60–100 μm) were calculated in all categories [7, 17, 19].

Density of Flk-1-Positive Cells and Immunoreactivity for VEGF and eNOS. In 20 non-overlapping randomly selected fields, the Flk-1-positive alveolar epithelial cells were counted (magnification ×400). This was limited to the air-exchanging parenchyma. The density of Flk-1-positive alveolar epithelial cells per square millimeter of tissue was recalculated. For Flk-1 immunoreactivity of the vascular smooth muscle and endothelial cells, and for VEGF and eNOS immunoreactivity of the lung tissue, semiquantitative analysis was performed using an arbitrary visual scale with grading score ranging from 0 to 4: grade 0 = no staining; grade 1 = focal staining, and grades 2, 3, and 4 = diffuse, weak, moderate and strong staining, respectively [20, 21].

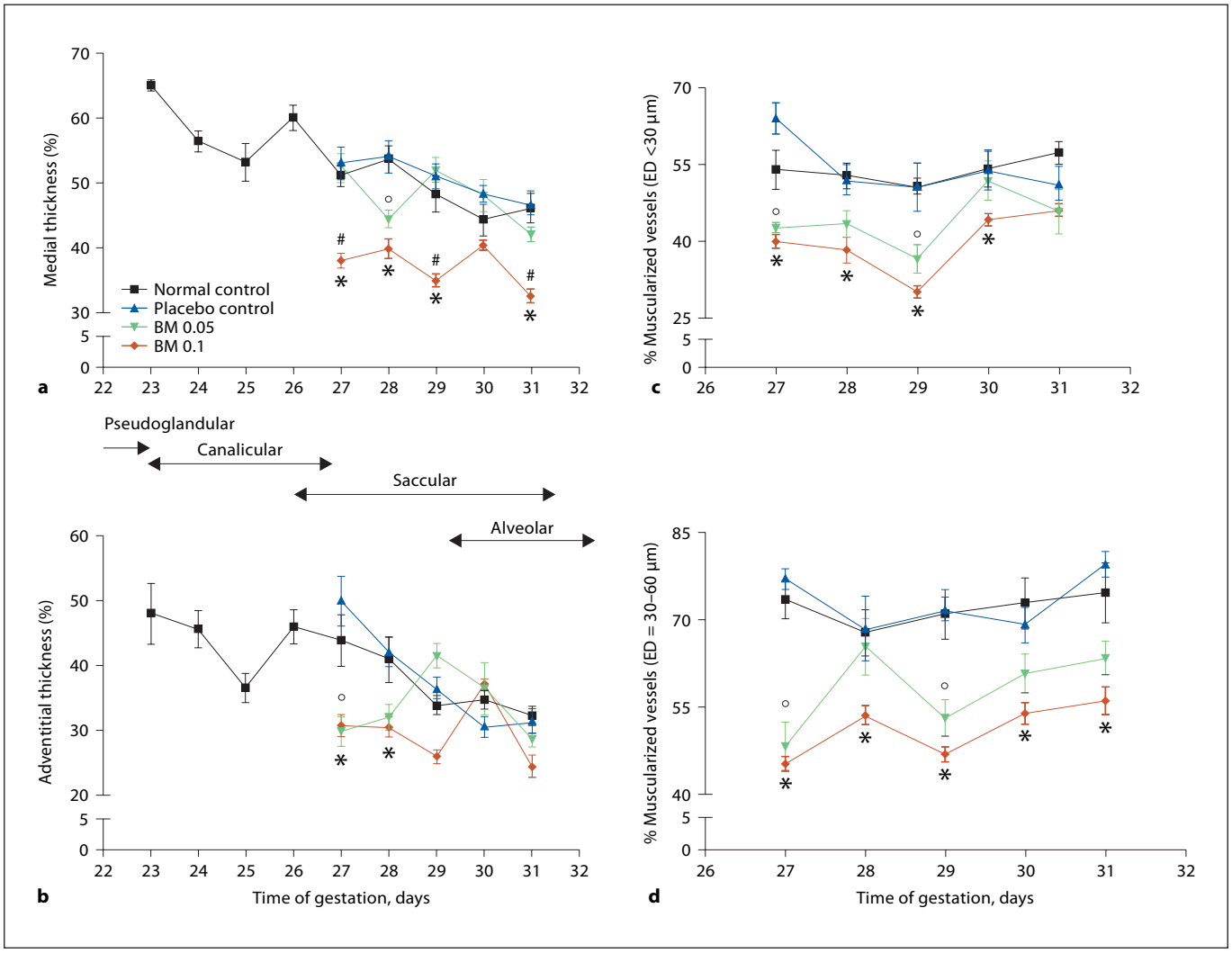


Fig. 1. Time course of changes in proportional medial thickness (**a**), proportional adventitial thickness (**b**), percentage of small (ED <30 µm, **c**) and intermediate (ED = 30–60 µm, **d**) muscularized pulmonary vessels after maternal administration of BM. Differences between ° BM = 0.05 and placebo, * BM = 0.1 and placebo, and # BM = 0.05 and BM = 0.1 were considered significant at $p < 0.05$.

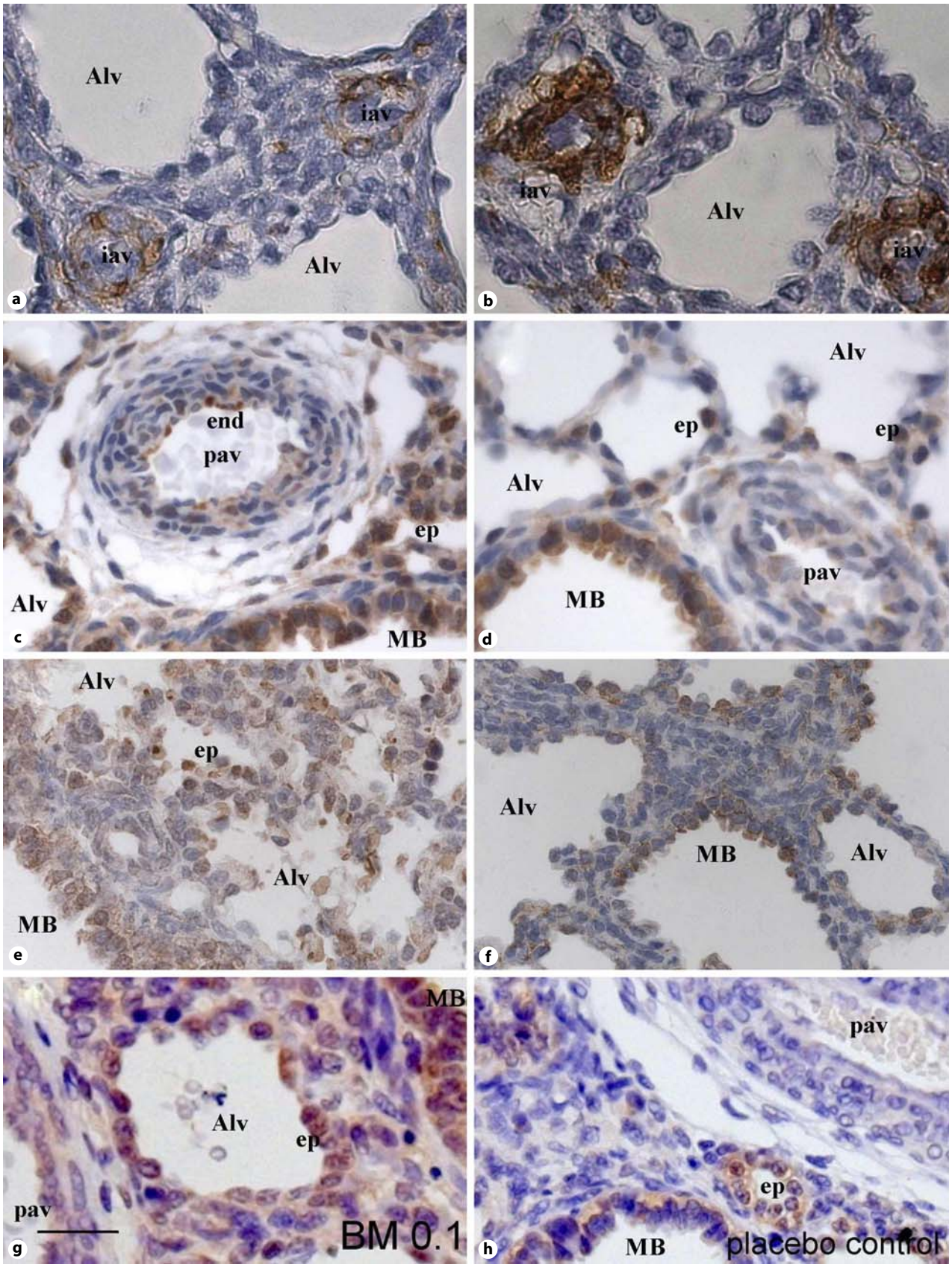
Statistical Analysis

Morphometric determinations were done by two observers (X.R. and AM.V.d.B.), who were blinded to the nature of the experimental procedure, and their observations were averaged. LBWR, morphometric measurements and derived calculations are presented as mean ± standard error of the mean (SEM). A day-by-day analysis over the pregnancy and within treatment groups was done with analysis of variance for multiple comparisons (ANOVA, Tukey's HSD test). The difference between BM groups and placebo at given time points was analyzed using independent t tests. All comparisons were performed using the Statistica 6.0 software (StatSoft, Inc., Tulsa, Okla., USA). Probability (p) values of <0.05 were considered statistically significant.

Results

After administration of BM, there was a significant decrease in FBW ($p = 0.003$) on day 27 and an even more prominent decrease in TLW (day 27, $p = 0.002$; day 28, $p = 0.001$), so that LBWR was significantly lower on day 27 for BM = 0.1 ($p = 0.001$) and on day 28 for BM = 0.05 ($p = 0.001$; table 1).

Changes in %MT and %AT demonstrate a progressive thinning of the vascular wall throughout normal gestation (days 23–31, $p = 0.001$; fig. 1a, b). Placebo fetuses showed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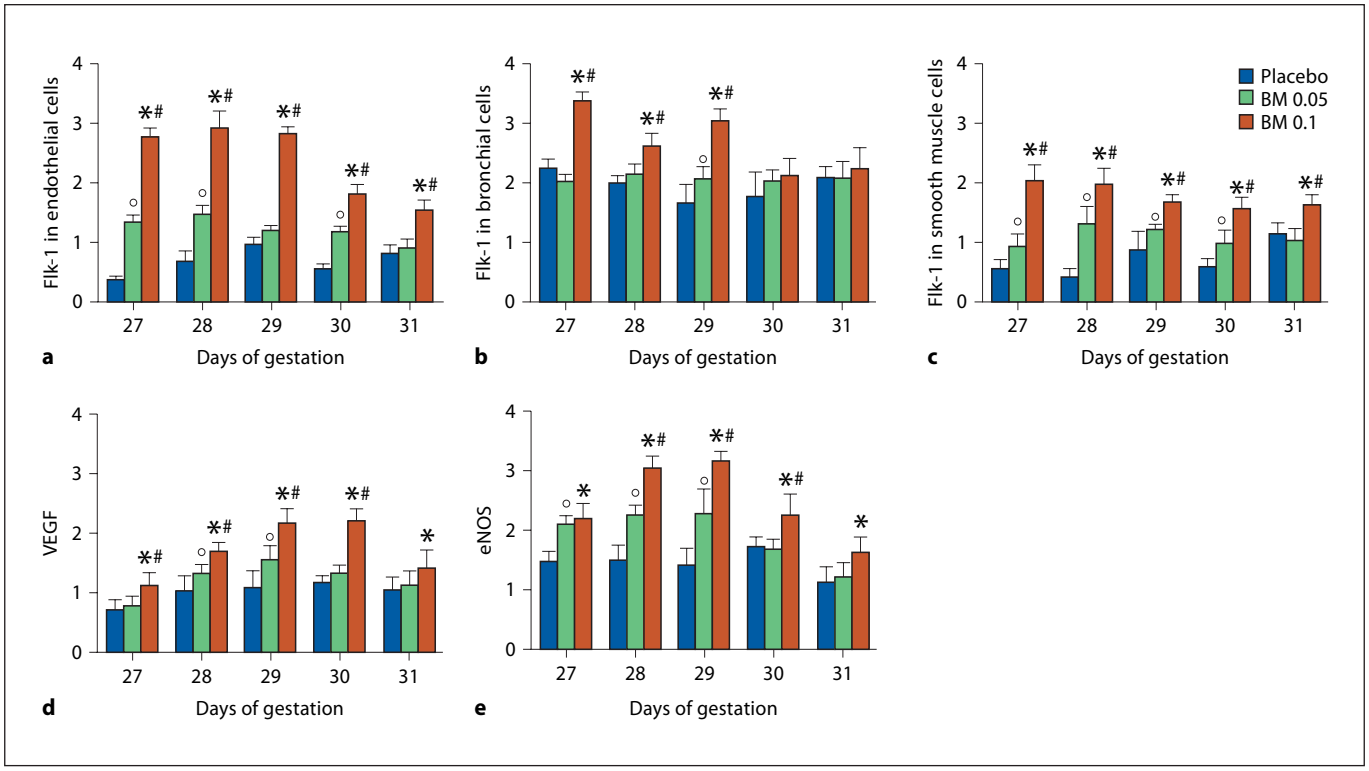

Fig. 3. Time course of changes in immunostaining grade of Flk-1 in endothelial cells (**a**), bronchial epithelium (**b**), vascular smooth muscle cells (**c**) and VEGF (**d**) and eNOS (**e**) in the total lung tissue after maternal administration of BM. Differences between ° BM = 0.05 and placebo, * BM = 0.1 and placebo, # BM = 0.05 and BM = 0.1 were considered significant at $p < 0.05$.

changes similar to controls. The effects of BM are dose-dependent. Following exposure to BM = 0.1, the %MT dropped significantly below that of the placebo group ($p = 0.001$), an effect which disappeared on day 30. For BM = 0.05 the decrease was only significant on day 28 ($p = 0.046$; fig. 1a). BM caused a decrease in %AT, its effect lasting longer after the higher dose (BM = 0.05: day 27, $p = 0.001$; BM = 0.1: day 27, $p = 0.001$; day 28, $p = 0.03$; fig. 1b).

In control fetuses, the number of smaller (ED <30 μm) muscularized vessels decreased between days 23 and 28 ($p = 0.02$) to remain unchanged later on. BM exerts a dose-dependent effect on the muscularization of small (ED <30 μm) and intermediate (ED = 30–60 μm) sized vessels (fig. 2a, b), the effect of BM = 0.1 (day 27, $p = 0.001$; day 28, $p = 0.001$; day 29, $p = 0.001$; day 30, $p = 0.01$) being more prominent and lasting longer than after BM = 0.05 (day 27, $p = 0.001$; day 29, $p = 0.04$; fig. 1c, d).

BM = 0.05 induced a 2-fold increase in Flk-1-positive alveolar epithelial cell density on day 27 ($p = 0.001$), whereas BM = 0.1 caused a 4-fold increase on days 27–28 ($p = 10^{-6}$, $p = 0.0003$) and a 2-fold increase until term (day 29, $p = 0.001$; day 30, $p = 0.001$; day 31, $p = 0.003$; table 1; fig. 2c, d, 4e, f). There was also increased Flk-1 immunoreactivity of the vascular endothelial cells (fig. 3a, 4a, b; $p = 0.002$), bronchial epithelial cells (fig. 3b; $p = 0.001$) and vascular smooth muscle cells (fig. 3c, 4a–d; $p = 0.001$) in a dose-dependent fashion. Further, BM increased the VEGF (fig. 2e, f, 3d; $p = 0.002$) and eNOS (fig. 2g, h, 3e; $p = 0.001$) expression as well; this effect also being dose-dependent.

Fig. 2. Fetal lung parenchyma (day 27), immunohistochemical sections counterstained with hematoxylin. Typical view of α-SMA (**a**, **b**), Flk-1 (**c**, **d**), VEGF (**e**, **f**) and eNOS (**g**, **h**) staining of lung parenchyma in BM = 0.1 and placebo group, respectively. Alv = Alveole; iav = intra-acinar vessels (ED <30 μm); pav = pre-acinar vessels (ED = 30–60 μm); MB = membrane bronchioles; ep = pulmonary epithelial cells; end = pulmonary vascular endothelial cells. Magnification ×640. Scale bar = 10 μm.

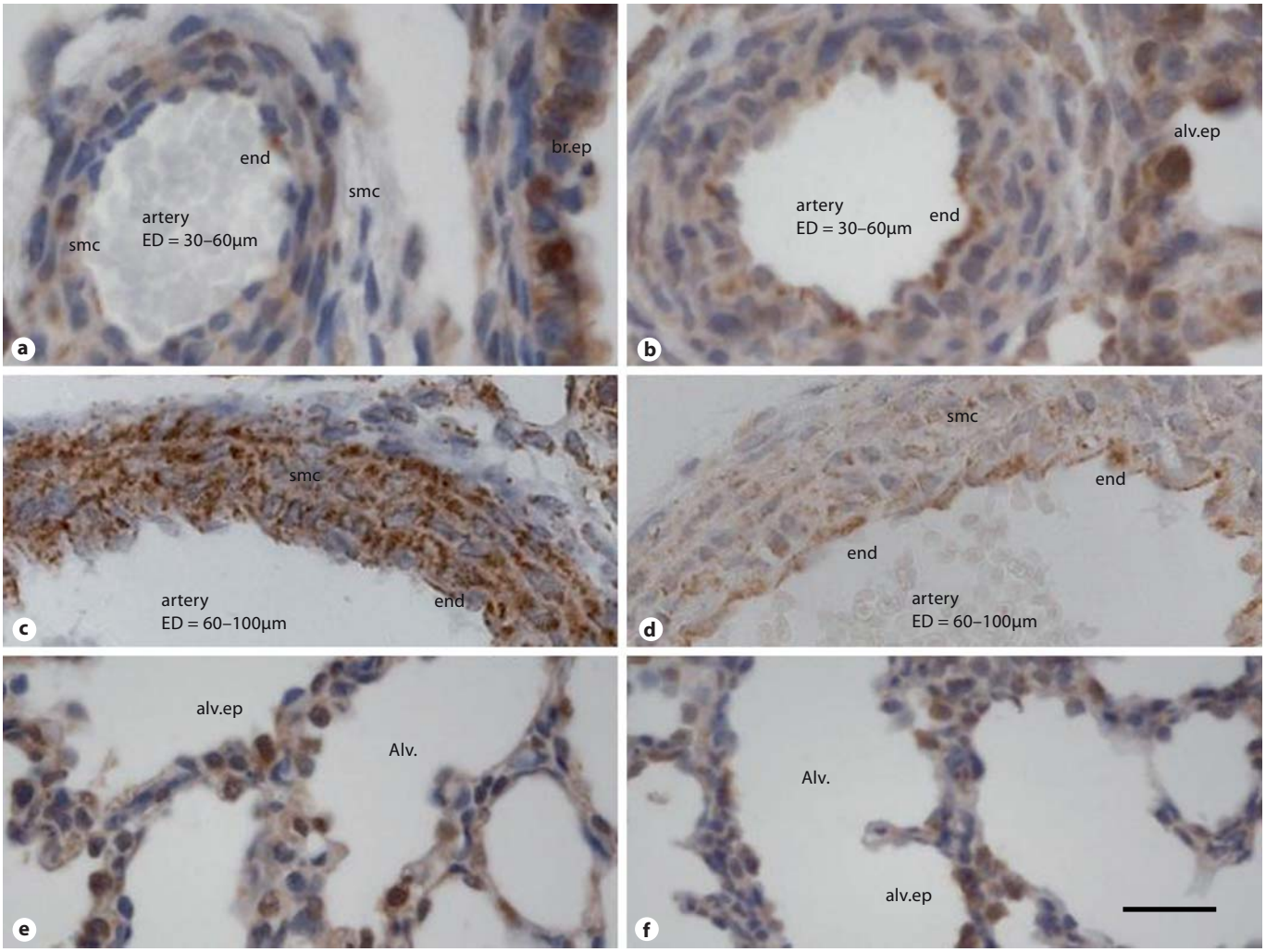

Fig. 4. Immunohistochemical staining for Flk-1 in intermediate pulmonary vessels (ED = 30–60 μm, **a**, **b**), in large pulmonary vessels (ED = 60–100 μm, **c**, **d**) and in the parenchyma (**e**, **f**) of a fetal rabbit lung (day 27) after maternal administration of BM = 0.1 or placebo, respectively. Alv. = Alveole; end = pulmonary vascular endothelial cells; smc = vascular smooth muscle cells; br.ep = bronchial epithelial cells; alv.ep = alveolar epithelial cells. Magnification ×640 (with use of a frame 768 × 512 pixels). Scale bar = 10 μm.

Discussion

Antenatal GC have been clinically used for several decades to enhance lung maturity, but they may reduce birth and lung weight [22]. GC reduce placental size, hence also fetal growth, potentially by decreasing IGF-II mRNA levels in junctional zones of the placenta [23]. This effect has been observed in fetal lambs [24] as well as rabbits [3]. A dose-effect relationship on lung and body weight has also been described earlier [2]. It is possible that the administered steroids mediate their effect on wet lung weight through decreased lung liquid production and/or reabsorption of lung liquid, as earlier documented in humans and rabbits [25, 37]. In the experiments of Tabor et al. [3], doses of 0.01, 0.03 and 0.1 mg/kg were compared, with the highest dose showing the most obvious effects. In our own experiments doses of 0.05 and 0.1 mg/kg/day were used without any effect on LBWR in term pups, but decreased LBWR when evaluated within 2 days after administration.

Adaptation to extra-uterine life requires a rapid increase in smooth muscle cell length and surface to increase pulmonary vessel lumen diameter and lower their resistance [26]. In persistent pulmonary hypertension of the newborn, pulmonary vasculature fails to relax at birth [1]. This is believed to be due to an immaturity in physiologic vascular remodeling. In clinical practice an improvement in cardiovascular adaptation is observed in premature neonates, who received antenatal GC [27]. Our work was mainly dedicated to the study of the time course of changes in peripheral lung vasculature following antenatal GC administration. We used an experimental model and morphometric techniques to document vascular thinning shortly after injection of BM in doses adapted to this model. Morphologically, the most important components of the media and adventitia are fibroblasts and smooth muscle cells. During angiogenesis in the pseudoglandular and canalicular stages, bronchial smooth muscle cells migrate and line up around the arteries. Later on, fibroblasts also migrate from the mesenchyme, line up around the arterial wall and develop smooth muscle cell phenotype [28]. Okoye et al. [29] earlier observed medial and adventitial thinning as well as a decrease in the percentage of muscularized intra-acinar blood vessels following antenatal administration of 0.25 mg/kg dexamethasone. This study, however, was in nitrofen-exposed rats that had congenital diaphragmatic hernias, and unfortunately no data on control fetuses (i.e. olive oil exposed) that received either saline or dexamethasone were included in the report [29]. The lungs of subjects with congenital diaphragmatic hernias are typically hypoplastic and show vascular changes on morphology.

In our study normally developing fetuses were exposed to BM, however also resulting in decreased medial and adventitial thickness. The proportion of muscularized small (ED <30 μm) and intermediate (ED = 30–60 μm) pulmonary arteries was decreased after BM administration as well. The observed effects were dose-dependent, i.e. more prominent and longer-lasting after the higher dose of BM = 0.1. Our study, its outcome variables and the methods used do not allow a direct explanation, so we can only speculate on the nature of our observations. Thinning of the media may be explained through inhibition of proliferation and migration of smooth muscle cells and/or fibroblasts. In vitro, dexamethasone has been shown to downregulate DNA synthesis, proliferation and chemotactic activity of human fetal lung fibroblasts in a dose-dependent fashion [30]. Dexamethasone further directly inhibits thrombin-stimulated proliferation of human airway smooth muscle cells [31] as well as proliferation, DNA synthesis [32] and migration of rat and human aortic smooth muscle cells in culture conditions [14].

VEGF is a potent vascular endothelial cell-specific mitogen. It stimulates angiogenesis, microvascular permeability, and causes vasodilatation. VEGF has been shown to induce release of NO from rabbit and human vascular endothelial cells [33] and to regulate eNOS expression [34]. Increased eNOS expression in neonatal rat lungs is also mediated by Flk-1 receptor activation. In fetal lambs, VEGF inhibition impaired endothelium-dependent vasodilatation, decreased eNOS expression and increased muscularization of small pulmonary arteries [35]. In our experiment, maternal administration of BM increased Flk-1-positive cell density in the alveolar parenchyma and Flk-1 immunoreactivity in vascular endothelial and smooth muscle cells. Immunoreactivity to VEGF and eNOS was also increased by BM administration. This makes a second, VEGF-dependent pathway for BM-induced suppression of smooth muscle cell, fibroblast proliferation and migration possible, if not likely.

Almost no data on the effects of GC on pulmonary vasculature have been published. In summary, we have shown that antenatal administration of BM on days 25–26 decreases the number of small muscularized intra-acinar vessels and leads to a medial and adventitial thinning of this category of vessels in normal rabbits by term. This suggests that antenatal steroid therapy may have a specific inhibitory effect on the developmental proliferation of the smooth muscle within the media of pulmonary arteries. Taking into account that GC induce an increased immunoreactivity for eNOS, VEGF and Flk-1, we can also suggest that antenatal GC may accelerate the normal process of medial wall attenuation in the pulmonary arteries, which usually occurs soon after birth. Our findings indicate that prenatal GC may reduce the risk of pulmonary hypertension developing in the lungs of premature neonates.

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