ANTIOXIDANTS & REDOX SIGNALING
Volume 6, Number 1, 2004

Forum Original Research Communication

Altered Expression of Cyclins and Cdks in Premature Infant Baboon Model of Bronchopulmonary Dysplasia

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ABSTRACT

Bronchopulmonary dysplasia (BPD) is a chronic lung disease of premature infants, which results in substantial morbidity. The pathophysiology of BPD includes oxidant injury, baro/volutrauma, and disordered lung repair. As lung development, differentiation, and repair require cell division, we hypothesized dysregulation of the cell cycle in oxygen exposure of premature infants that may contribute to the evolution of BPD. In this investigation, we studied the expression of cyclins and cyclin-dependent kinases (cdks) that regulate transition from G1 and G2 phases of the cell cycle. We report here that expression of cyclin D1, cyclin E, and cyclin A is modulated in premature baboons in respiratory distress. In addition, the expression of cdk1 or cdk4 was also modulated in these premature animals. The phosphorylation of retinoblastoma protein was progressively decreased in 125-day animals and in 140-day animals exposed to 6 or 14 days of PRN oxygen. These results indicate that due to altered cyclin and cdk expression, the repair of injured epithelium may proceed in a disordered manner that is characteristic of BPD. Thus, altered cell cycle regulation may be an important factor in the evolution of BPD. *Antioxid. Redox Signal.* 6, 117–127.

INTRODUCTION

BRONCHOPULMONARY DYSPLASIA (BPD) is a chronic lung disease (CLD) of premature infants, which results in substantial morbidity (8, 9). The pathophysiology of BPD involves oxidant injury, baro/volutrauma, and disordered lung repair, all of which result in poor alveolization and disrupted microvascular development (20). Elevated oxygen tension can elicit DNA damage and growth arrest of lung cells in various cell culture models (for review, see 23), and oxidative insult to the developing lung may disrupt the orderly pattern of proliferation. Few studies have examined the pattern of cell proliferation under conditions of increased oxygen tension, but exposure to high concentrations of oxygen is generally associated with decreased proliferation of endothelial, interstitial, type I and type II pneumocytes (29). However, when several species of adult animals were exposed to 100% oxygen and allowed to recover for 7 days, the cell numbers increased (29). Increased type II cell proliferation was noted during the recovery phase following exposure to hyperoxia (29). In addition to an inhibited proliferative response, monkeys exposed to 95% oxygen for 14 days had complete destruction of type I cells (15). A rapid proliferation of type II alveolar cells is crucial for proper healing after injury, because delayed reepithelialization may lead to the development of pulmonary fibrosis (2, 5).

The cell cycle is the period during which events required for successful cell reproduction are completed (23, 25). The progression of the cell cycle is a highly ordered process that is tightly coupled to correct duplication of the genetic material. Checkpoint control mechanisms have evolved to ensure correct passage of the genetic material to daughter cells (30). If the genetic material is altered, these control mechanisms halt the cell cycle in order to repair the genetic material. Failure of cycle correction and repair ultimately drives damaged cells toward apoptotic death (14, 30).

Progression of cells through the G1 phase and the G1/S transition involves sequential assembly and activation of key regulators of the cell cycle machinery, the cyclin-dependent kinases (cdks). The kinase activity of all cdks requires binding of a positive regulatory subunit known as cyclin (25). Each

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phase of the cell cycle is characterized by the expression of a distinct type of cyclin, and fluctuations in cyclin levels are the primary means of regulating cdk activity. D-type cyclins (D1, D2, and D3) are the first to be expressed when quiescent cells are stimulated by growth factors (27). At least one type of cyclin D is needed to complete the G1 phase. D-type cyclins associate with cdk4 or cdk6 to form complexes whose major substrate is retinoblastoma protein (Rb) and related proteins, p107 and p130 (3, 18). Quiescent cells contain a complex of hypophosphorylated Rb and p130 with transcription factor E2F (19, 25). Phosphorylation of Rb releases transcription factors, E2Fs, and activates transcription. Target genes for E2Fs are those required for G1/S phase transition, as well as those genes necessary for DNA replication. Although expression of D-type cyclins has been recorded in cell cultures (1), and in mouse models of hyperoxia (1) their expression in BPD has not been investigated.

The next cyclin to be expressed is cyclin E that complexes with cdk2; the resulting kinase activity is required for S-phase entry and the initiation of the DNA replication (23). Cyclin E-cdk2 phosphorylates the Rb family of proteins at sites different from cyclin D-cdk4–6 (10, 23), and this dual phosphorylation appears to be required for full activation of the Rb proteins. Cyclin E-cdk2 also phosphorylates and targets the cdk inhibitor p27 (10) for degradation, as well as cyclin E itself (10). Studies in transformed cell lines indicated that hyperoxia increased cyclin E expression (10). Despite increased expression of cyclin subunit, hyperoxia significantly decreased the activity of cyclin E-cdk2 in these cells (10).

The central and rate-limiting function in the transition from G2 into the M phase depends on a highly conserved protein kinase (19). This complex is known as maturation promoting factor (MPF), and its activation is essential for cells to enter mitosis (19). MPF is composed of two components, the cyclin B1 and the kinase catalytic subunit $p34^{cdc2}$ (the homologue of yeast cdc2 gene, also known as cdk1). Threshold levels of cyclin B1 synthesis and dephosphorylation of Tyr-15 and Thr-14 residue of $p34^{cdc2}$ mediate the activation of $p34^{cdc2}$ by cdc25 phosphatase (19). Although the response of $p34^{cdc2}$ has been shown to increase in fetal mouse lung tissue and in the recovery period following exposure to oxygen, the expression of cyclin B1 has been shown to decrease in cultured lung cells.

In this investigation, we evaluate the expression of important cell-cycle regulatory cyclins and cdks, and demonstrate that the expression of these molecules are modulated in the premature infant model of BPD, which suggests that cell division and differentiation may be compromised. Thus, cell-cycle alterations may be facilitating the disordered lung architecture and repair characteristics of BPD.

MATERIALS AND METHODS

Animal studies

All animal care procedures were performed according to the National Research Council's Guide for the Care and Use of Laboratory Animals. Protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Southwest Foundation for Biomedical Research, San Antonio, Texas. Fetal baboons of varying gestational ages (±2 days) were delivered by hysterotomy. Gestational ages were determined by timed matings as previously described (12, 13) with confirmation by ultrasound at intervals during pregnancy.

In other studies pertinent to the effect of variable oxygen tension in vivo and to the development of BPD following hyaline membrane disease, treatment groups were delivered at either 140 ± 2 days or 125 ± 2 days of gestation and immediately placed on positive pressure ventilation. Animals of 140-day gestation were given either continuous 100% oxygen or an inspired oxygen tension as needed [pro re nata (PRN)] to maintain paO_2 at 40–50 torr. Within 10 days, those 140-day animals given 100% oxygen develop lung histopathologic lesions that closely resemble human BPD, whereas the PRN animals do not develop these lesions, allowing near-normal lung development (16, 28). More premature animals of 125-day gestation received immediate resuscitation with artificial surfactant, positive pressure ventilation, and PRN oxygen. These animals also develop BPD despite receiving lesser (PRN) inspired concentrations of oxygen (28). In either case, all animals received state-of-the-art care in a neonatal intensive care unit for up to 17 days. Following treatment, animals were killed by administration of intravenous pentobarbital. The lungs were perfused via the pulmonary artery with phosphate-buffered saline (37°C), and distal lung tissue was dissected free from major airways and central structures and processed immediately as for fetal tissue. The number of animals that were exposed to hyperoxic regimen or gestational controls (GCs) varied between three and four. The paraffin-embedded slides were received from the BPD Resource Center from one or more animals from the same experimental group.

Immunohistochemistry

Tissue sections were deparaffinized in xylene and rehydrated through graded mixtures of ethanol and deionized water. Immunostaining was done with either a Santa Cruz immunoperoxidase staining kit or a Dako universal ABC. Endogenous peroxidase was quenched with the peroxidase quench solution provided with the kit. All sections were blocked for nonspecific binding with serum and then incubated with primary antibody overnight at 4°C. Monoclonal or polyclonal antibodies to cyclin A, D, E, and B1, Rb, and cdk4 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Phospho-specific antibody to Rb and $p34^{cdc2}$ was obtained from Cell Signaling Technologies (Beverly, MA, U.S.A.). The slides were then rinsed in phosphate-buffered saline and incubated with a biotinylated secondary antibody followed by incubation with streptavidin conjugated with horseradish peroxidase. Immunoreactivity was detected with an aminoethylcarbazole kit (Sigma), which develops the reaction as a reddish or brown color. The sections were then counterstained with hematoxylin and mounted in aqueous media, and the slides were observed and digitally photographed with an Olympus microscope.

RESULTS

Increased expression of cyclin D1 in premature infant baboons with BPD

When cells transit from G0 to G1 in the cell cycle, the D-type cyclins (D1, D2, and D3) are induced before cyclins A and E. At least one type of cyclin D is needed to complete the

G1 phase (5). D-type cyclins assemble with cdk4 or cdk6 to form complexes whose major substrate is Rb along with related proteins, p130 and p107. Growth factors maintain D-type cyclin at relatively constant levels throughout the cell cycle. As D-type cyclins are not expressed in quiescent cells (23), the appearance of these markers indicates cell proliferation. To assess the level of D-type cyclin in premature baboons with BPD, we detected cyclin D1 by immunohistochemistry using an anti-cyclin D1 monoclonal antibody. As shown in Fig. 1, exposure to oxygen for either 6 or 14 days PRN increased cyclin D1 in the lungs of 125-day infants compared with their GCs. In addition, 140-day animals also demonstrated increased immunostaining for cyclin D1 after 6 days of PRN or 6 days of 100% oxygen exposure. However, the level of immunostaining was less than that of 125-day animals exposed to 6 or 14 days of PRN oxygen.

Modulation of cyclin E expression in premature infant baboons with BPD

Cyclin E complexes with cdk2, and the resulting kinase activity is required for entry of cells into S-phase and initiation of DNA replication. Therefore, we examined the expression of cyclin E in lungs of premature infants with BPD. As demonstrated in Fig. 2, the level of cyclin E was increased in 125-day animals exposed to 6 days of PRN oxygen compared with the GC animals (Fig. 2, upper panels). However, there was a decrease in the localization of cyclin E in lungs of baboons exposed to 14 days of PRN oxygen. Similarly, 140-day animals exposed to 6 days of PRN oxygen had a marginal increase in the expression of cyclin E compared with 140-day GC animals. In contrast, 140-day animals exposed to 100% oxygen for 6 days had a decreased level of cyclin E expression.

Modulation of cyclin A expression in premature baboons with BPD

Cyclin A is expressed soon after cyclin E at the G1/S boundary and also forms complexes with cdk2 and to a lesser extent with cdc2 (5). The activity of cyclin A-cdk2 is required for S-phase transition and control of DNA replication (5). Therefore, we evaluated the expression of cyclin A in premature infant baboons with BPD. As demonstrated in Fig. 3, 125-day GC animals had abundant expression of cyclin A. Exposure of these animals to either 6 or 14 days of PRN oxygen decreased the level of cyclin A expression in 125-day animals. Similarly, 140-day GC animals had a high level of cyclin A expression that was progressively reduced following exposure to 6 days of PRN or 6 days of 100% oxygen.

Modulation of cdk4 expression in premature baboons with BPD

Kinase activity of all Cdks requires the binding of cyclins. Cyclin D, cyclin E, and cyclin A form complexes with cdk4 at various phases of the cell cycle. Therefore, we determined whether the level of cdk4 is modulated in BPD. As demonstrated in Fig. 4, the expression of cdk4 was increased in 125-day animals exposed to 6 days of PRN oxygen. However, the level was decreased in 14-day PRN oxygen-exposed animals (Fig. 4, upper panels). In a similar manner, 140-day animals had increased cdk4 expression in 6-day PRN animals, but the level of cdk4 was decreased in 140-day animals exposed to 6 days of 100% oxygen (Fig. 4, lower panels).

Decreased expression of Rb in premature baboons with BPD

Critical substrates of the G1 cyclin-cdk complexes include the Rb and related proteins, p107 and p130 (5). D-type cyclins can bind directly to Rb and target cdk4 to this protein (10). The hypophosphorylated form of Rb binds the E2F family of transcription factors. Phosphorylation of Rb by kinases (cyclin D-cdk4 kinase and cyclin E-cdk2 kinase) releases E2Fs, enabling them to activate genes required for DNA replication. Rb phosphorylation appears to be triggered first by the D-type cyclin-cdk complex, and then by the cyclin E-cdk2 complex. The onset of Rb phosphorylation induces up-regulation of E2F and cyclin E synthesis, which, in turn, increases cyclin E-cdk2 complexes. Rb phosphorylation is required for progression through the restriction point late in G1, and as the level of Rb is crucial to completing this step, we measured levels of Rb protein in lung tissue from 125-day and 140-day baboons with BPD. Figure 5 shows that 125-day GC animals had abundant Rb protein, which was progressively reduced in oxygen-treated animals (6 or 14 days PRN). Oxygen exposure (6 days of PRN or 6 days of 100% oxygen) of the 140-day gestational age animals also resulted in decreased Rb levels compared with 125-day gestational age animals. These studies suggest that extremely premature as well as premature baboons with BPD may experience inappropriate cell-cycle regulation and cell-cycle arrest.

Modulation of phosphorylation of Rb in premature baboons with BPD

Phosphorylation of Rb is performed by cyclin D-cdk4 and cyclin E-cdk2 complexes at different residues (27). As phosphorylation of Rb is a crucial step for progression of the cell cycle through the G1 restriction point, we sought to determine the level of Rb phosphorylation in BPD. As demonstrated in Fig. 6, the level of Rb phosphorylation was increased in 125-day animals exposed to 6 days of PRN oxygen and remain elevated after 14 days of PRN exposure (Fig. 6, upper panels). Similarly, in 140-day animals, the phospho-Rb levels increased in 6-day PRN oxygen-exposed animals, but decreased in 14-day 100% oxygen-exposed animals (Fig. 6, lower panels).

Modulation of cyclin B1 expression in premature baboons with BPD

The expression of cyclin B1 peaks at the end of G2 phase. The threshold level of cyclin B1 is synthesized and complexes with $p34^{cdc2}$, resulting in the activation of the kinase activity of the complex. This kinase activity is required for the progression of cell cycle to mitosis. Therefore, we evaluated the expression of cyclin B1 in premature infant baboons with BPD. As demonstrated in Fig. 7, the expression of cyclin B1 increased in animals exposed to 6 days as well as 14 days of PRN oxygen compared with 125-day GC (Fig. 7, upper panels). In the 140-day animals, the expression of cyclin B1 was increased in 6-day PRN oxygen-exposed animals, but decreased in 6-day 100% oxygen-exposed animals.

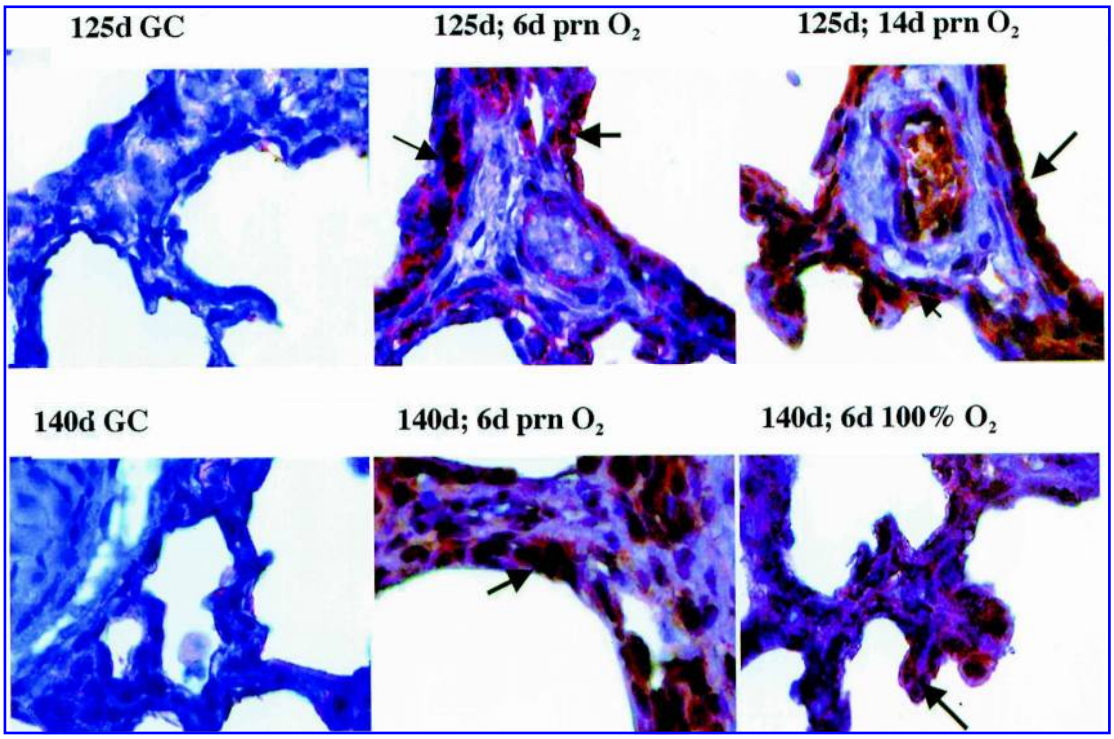

FIG. 1. Increased expression of cyclin D1 in premature infant baboons with BPD. Paraffin-embedded baboon lung tissue sections were processed for cyclin D1 immunohistochemistry as described in Materials and Methods. Red or brown color (arrow) indicates localization of cyclin D1. **(Upper panels)** 125-day GC and 125-day animals exposed to 6 days and 14 days of PRN oxygen. **(Lower panels)** 140-day GC and 140-day animals exposed to 6 days of PRN oxygen and 6 days of 100% oxygen.

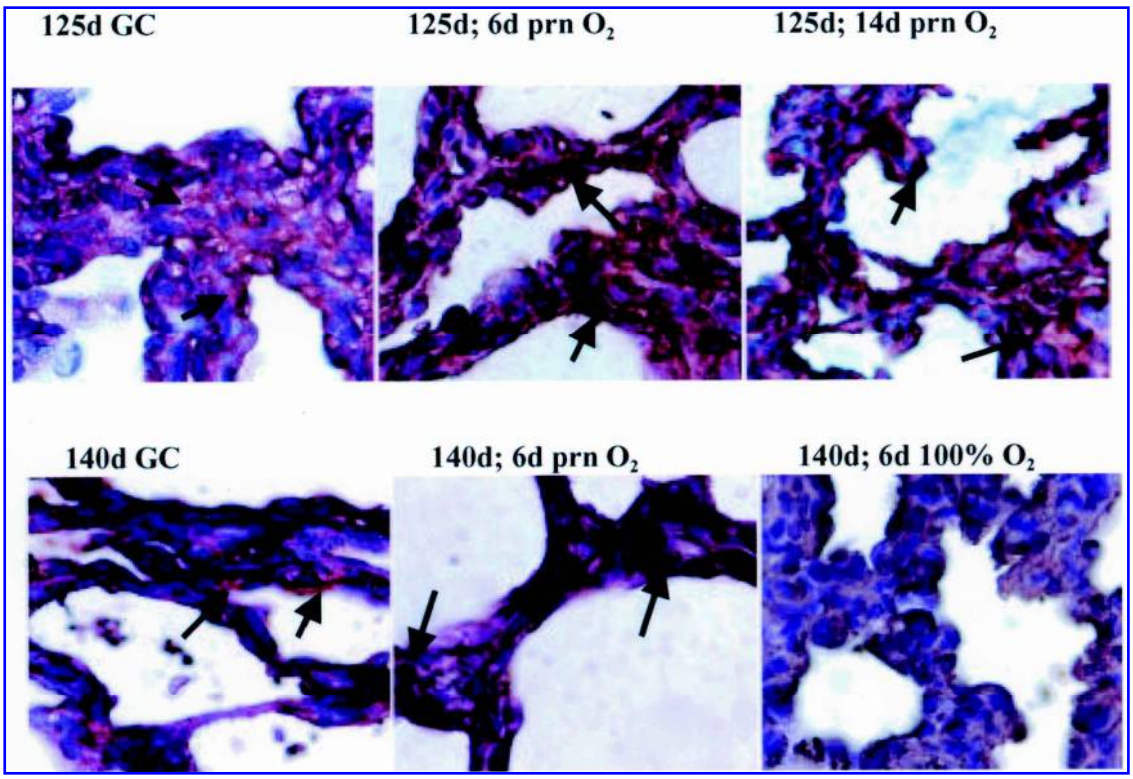

FIG. 2. Modulation of cyclin E expression in premature infant baboons with BPD. Paraffin-embedded baboon lung tissue sections were processed for cyclin E immunohistochemistry as described in Materials and Methods. Red or brown color (arrow) indicates localization of cyclin E. **(Upper panels)** 125-day GC and 125-day animals exposed to 6 days and 14 days of PRN oxygen. **(Lower panels)** 140-day GC and 140-day animals exposed to 6 days of PRN oxygen and 6 days of 100% oxygen.

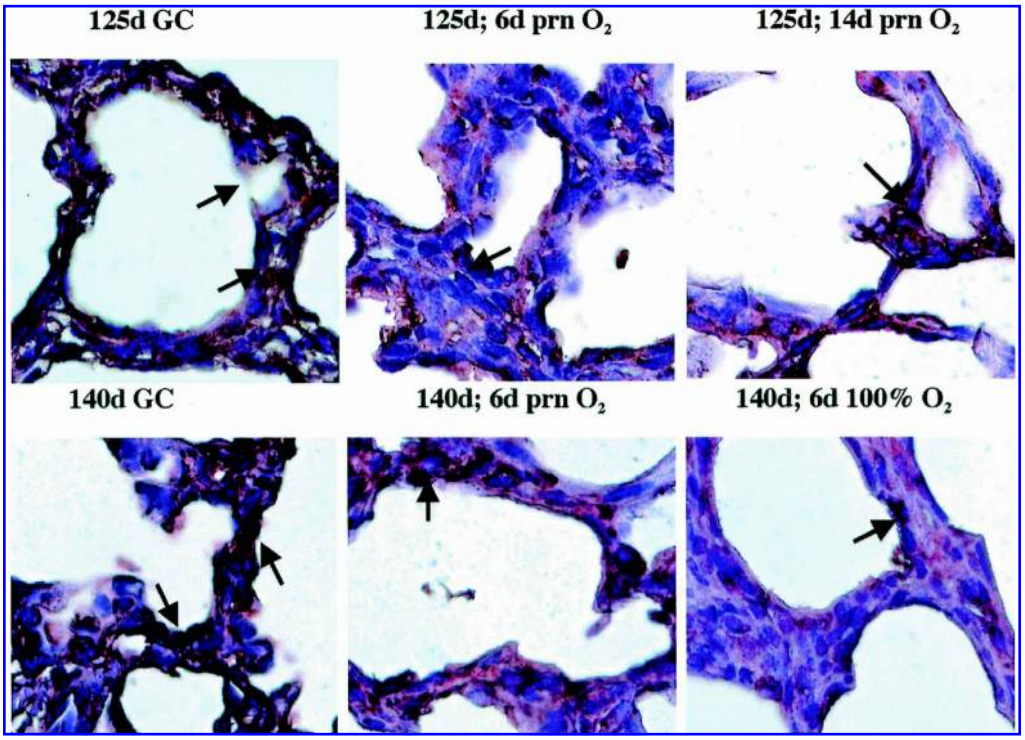

FIG. 3. Modulation of cyclin A expression in premature baboons with BPD. Paraffin-embedded baboon lung tissue sections were processed for cyclin A immunohistochemistry as described in Materials and Methods. Red or brown color (arrow) indicates localization of cyclin A. **(Upper panels)** 125-day GC and 125-day animals exposed to 6 days and 14 days of PRN oxygen. **(Lower panels)** 140-day GC and 140-day animals exposed to 6 days of PRN oxygen and 6 days of 100% oxygen.

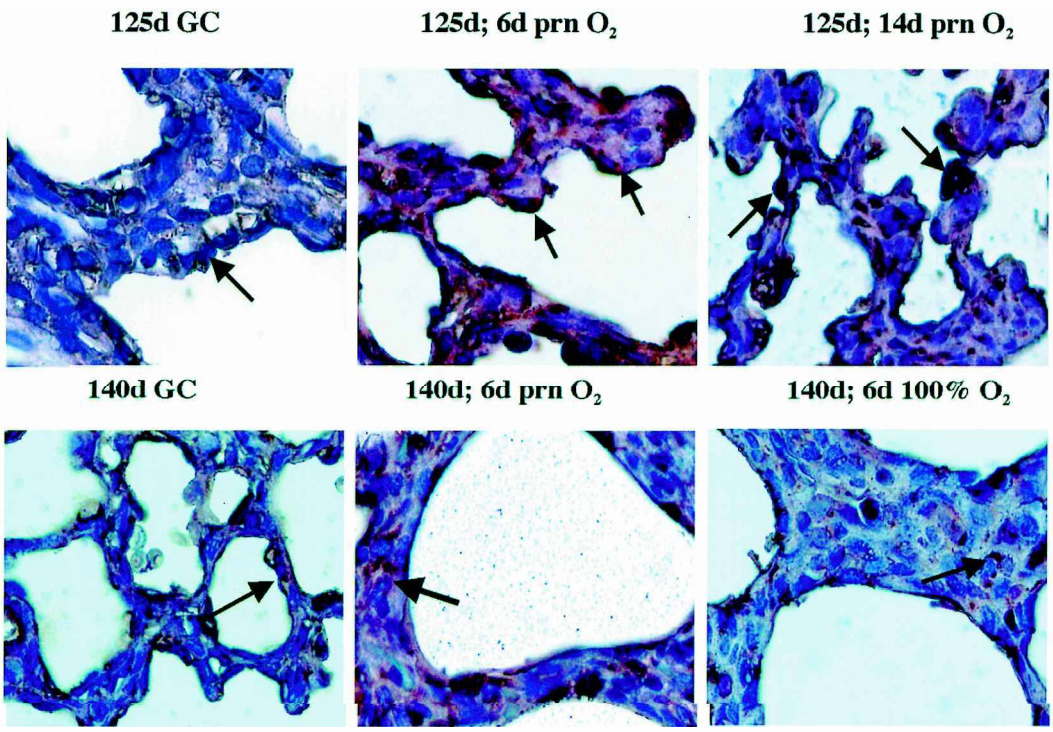

FIG. 4. Modulation of cdk4 expression in premature baboons with BPD. Paraffin-embedded baboon lung tissue sections were processed for cdk4 immunohistochemistry as described in Materials and Methods. Red or brown color (arrow) indicates localization of cdk4. **(Upper panels)** 125-day GC and 125-day animals exposed to 6 days and 14 days of PRN oxygen. **(Lower panels)** 140-day GC and 140-day animals exposed to 6 days of PRN oxygen and 6 days of 100% oxygen.

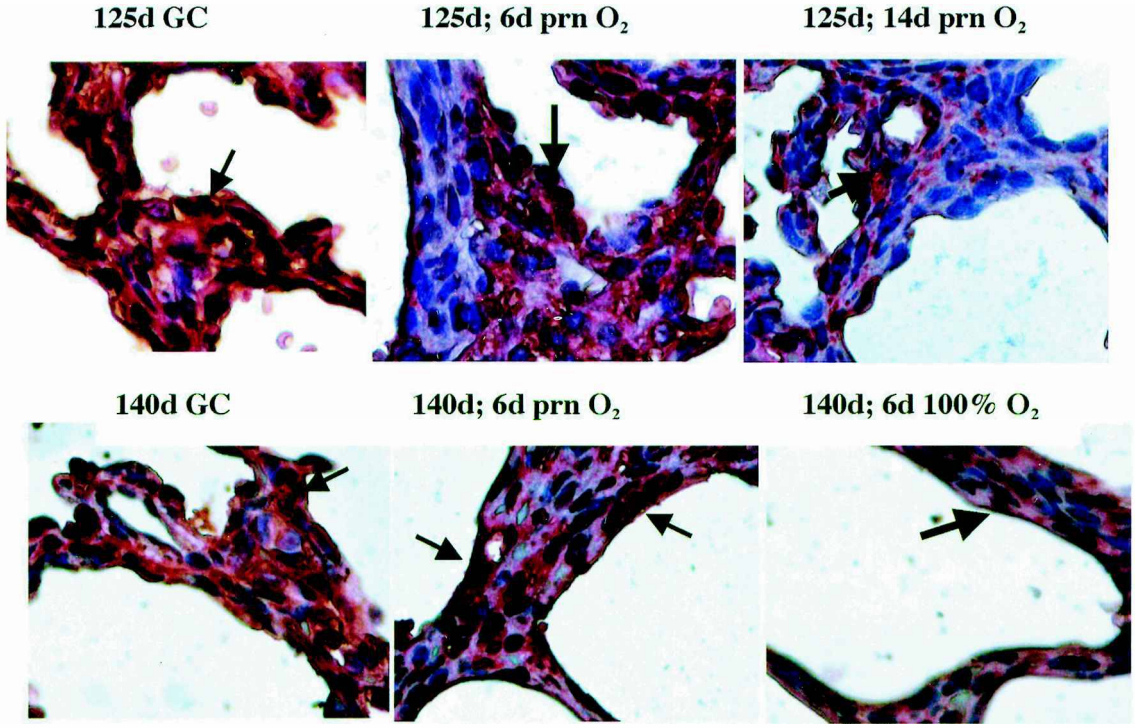

FIG. 5. Decreased expression of Rb in premature baboons with BPD. Paraffin-embedded baboon lung tissue sections were processed for Rb immunohistochemistry as described in Materials and Methods. Red or brown color (arrow) indicates localization of Rb. **(Upper panels)** 125-day GC and 125-day animals exposed to 6 days and 14 days of PRN oxygen. **(Lower panels)** 140-day GC and 140-day animals exposed to 6 days of PRN oxygen and 6 days of 100% oxygen.

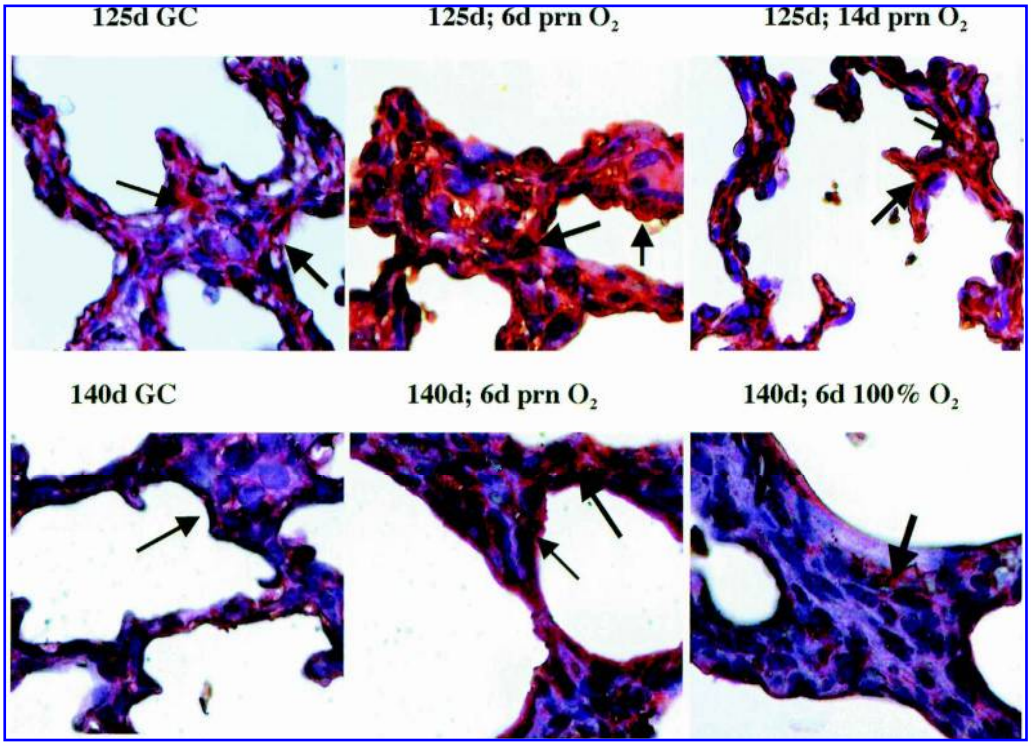

FIG. 6. Modulation of phosphorylation of Rb in premature baboons with BPD. Paraffin-embedded baboon lung tissue sections were processed for phospho-Rb immunohistochemistry as described in Materials and Methods. Red or brown color (arrow) indicates localization of phospho-Rb. **(Upper panels)** 125-day GC and 125-day animals exposed to 6 days and 14 days of PRN oxygen. **(Lower panels)** 140-day GC and 140-day animals exposed to 6 days of PRN oxygen and 6 days of 100% oxygen.

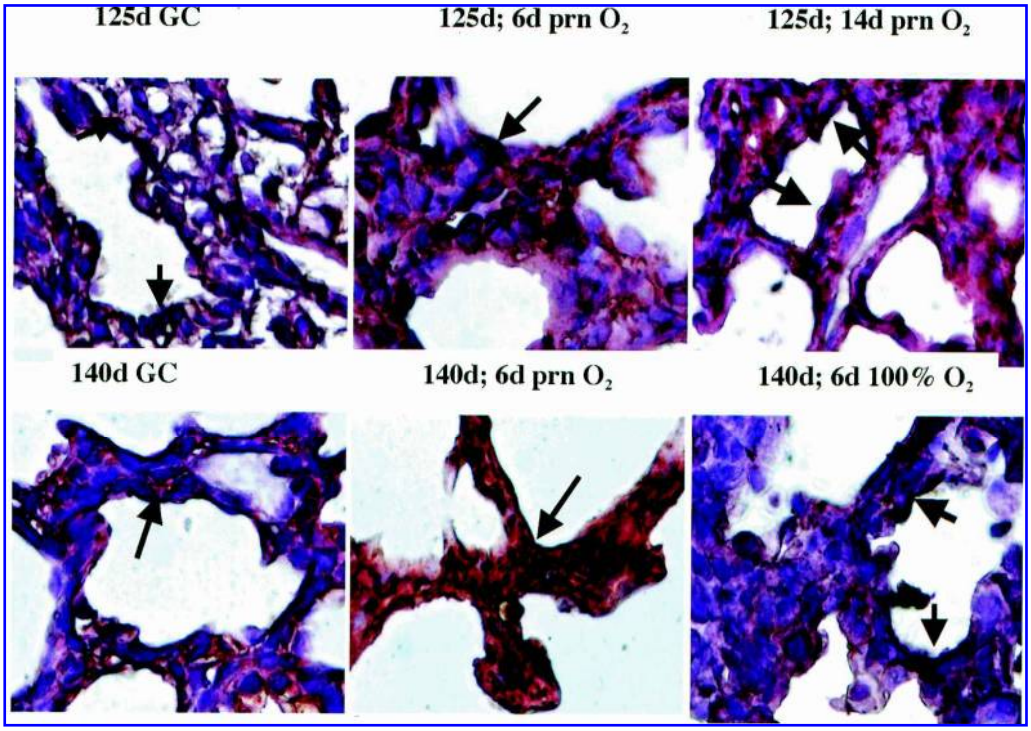

FIG. 7. Modulation of cyclin B1 expression in premature baboons with BPD. Paraffin-embedded baboon lung tissue sections were processed for cyclin B1 immunohistochemistry as described in Materials and Methods. Red or brown color (arrow) indicates localization of cyclin B1. **(Upper panels)** 125-day GC and 125-day animals exposed to 6 days and 14 days of PRN oxygen. **(Lower panels)** 140-day GC and 140-day animals exposed to 6 days of PRN oxygen and 6 days of 100% oxygen.

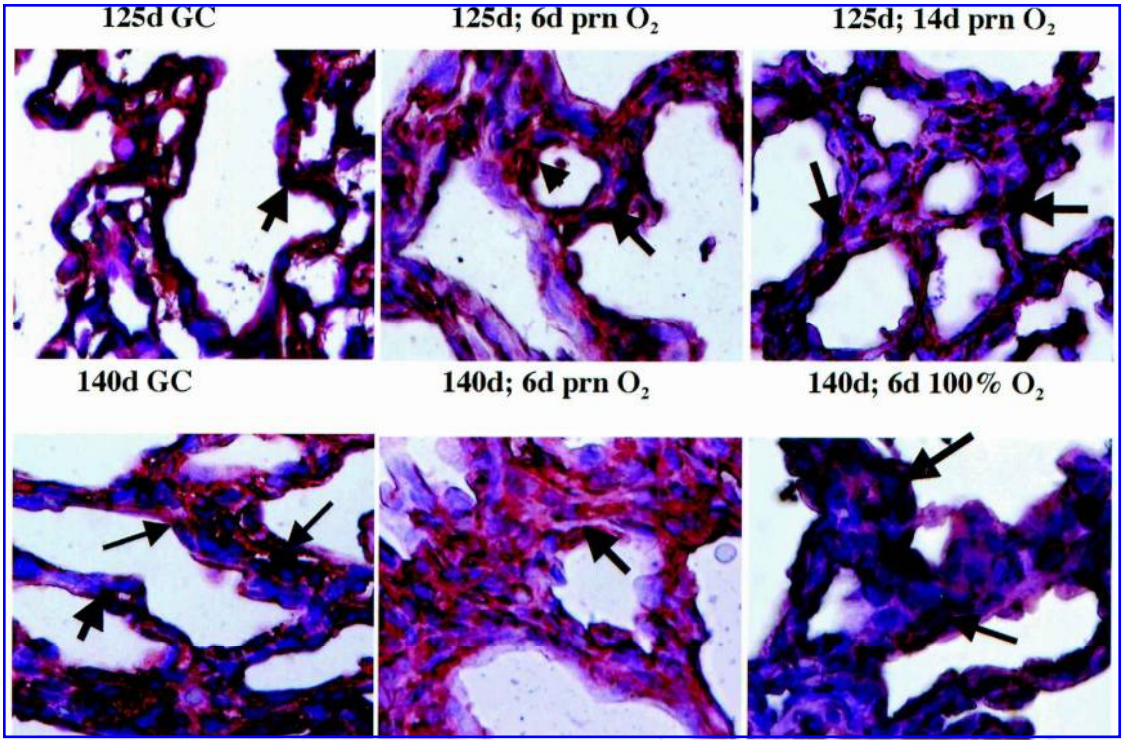

FIG. 8. Modulation of $p34^{cdc2}$ expression in premature baboons with BPD. Paraffin-embedded baboon lung tissue sections were processed for $p34^{cdc2}$ immunohistochemistry as described in Materials and Methods. Red or brown color (arrow) indicates localization of $p34^{cdc2}$. **(Upper panels)** 125-day GC and 125-day animals exposed to 6 days and 14 days of PRN oxygen. **(Lower panels)** 140-day GC and 140-day animals exposed to 6 days of PRN oxygen and 6 days of 100% oxygen.

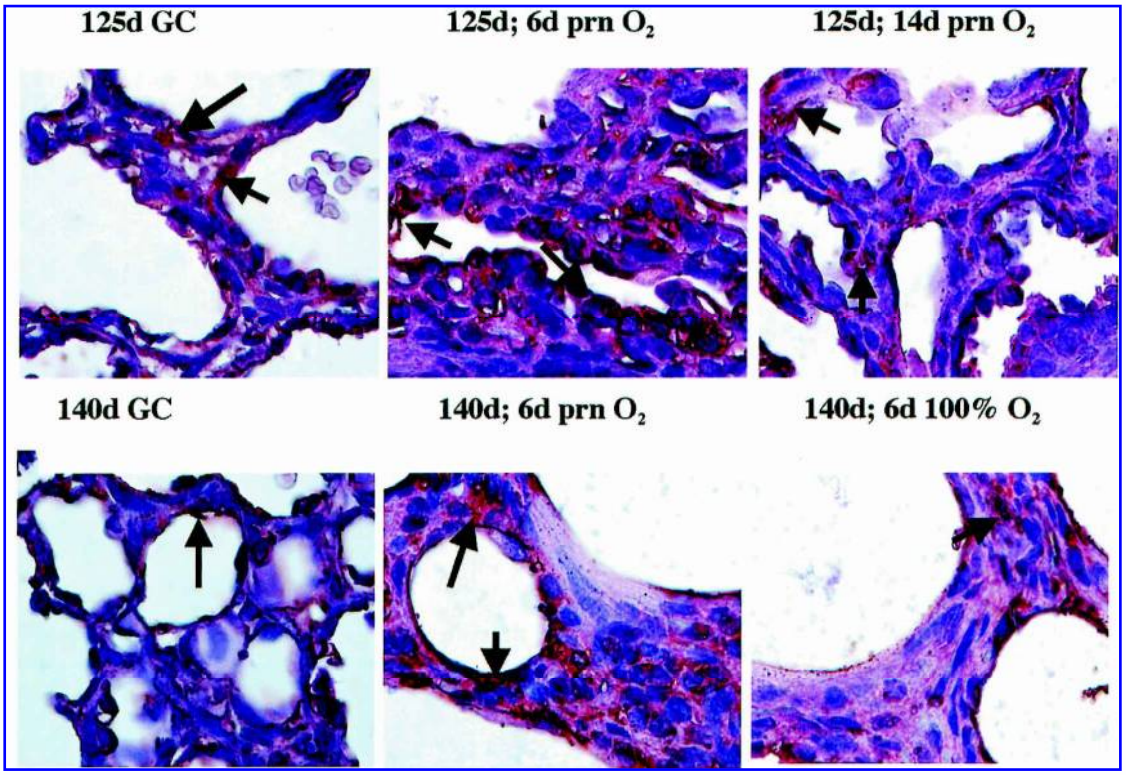

FIG. 9. Modulation of p34^{cdc2} phosphorylation in premature baboons with BPD. Paraffin-embedded baboon lung tissue sections were processed for phospho-p34^{cdc2} immunohistochemistry as described in Materials and Methods. Red or brown color (arrow) indicates localization of phospho-p34^{cdc2}. **(Upper panels)** 125-day GC and 125-day animals exposed to 6 days and 14 days of PRN oxygen. **(Lower panels)** 140-day GC and 140-day animals exposed to 6 days of PRN oxygen and 6 days of 100% oxygen.

Modulation of p34^{cdc2} expression in premature baboons with BPD

Most lung cell types undergo active proliferation during lung development. p34^{cdc2} is a rate-limiting protein that drives cells to mitosis, and increased expression of p34^{cdc2} has been observed in fetal rat lung (1, 31). For this study, we analyzed baboon lung samples for p34^{cdc2} expression as a means of determining whether p34 levels are modulated in BPD. As expected, we found that 125-day or 140-day GCs had a high level of p34^{cdc2} expression, indicating cell proliferation in the fetal lung. Exposure of premature infant baboons to oxygen for 6 days PRN did not alter expression of p34^{cdc2}; however, exposure to 100% O_2 decreased expression (Fig. 8) of p34^{cdc2}.

Modulation of p34^{cdc2} phosphorylation in premature baboons with BPD

The kinase activity of p34^{cdc2} is activated by dephosphorylation of Tyr-15 and Thr-14 residues by cdc25C phosphatase (17). Therefore, increased phosphorylation of p34^{cdc2} would indicate inactivation of p34^{cdc2}. As the kinase activity of cyclin B1-p34^{cdc2} complex is dependent on the phosphorylation status of p34^{cdc2}, we examined the phosphorylation state of p34^{cdc2} in premature infant baboons in BPD. As demonstrated in Fig. 9, increased phosphorylation of p34^{cdc2} was observed in 125-day animals exposed to 6 days of PRN oxygen (Fig. 9, upper panels). However, 125-day animals exposed to 14 days of PRN oxygen have decreased phosphorylation of p34^{cdc2}. Additionally, 140-day animals exposed to 6 days of PRN oxygen had increased phosphorylation of p34^{cdc2} compared with GC animals. Animals exposed to 100% oxygen had less phosphorylation of p34^{cdc2}.

DISCUSSION

Studies relating to the regulation of G1 or G2 cell-cycle progression in oxidative stress conditions including hyperoxia have been performed mainly in cell culture models using a variety of cell types (4, 5, 10, 21, 22). In most studies, hyperoxia is synonymous with 95% oxygen exposure. Thus, although there is a paucity of studies using lesser levels of oxygen in cell culture models, studies involving response of premature infant animals to oxygen are also lacking. The present study using a premature baboon BPD model is an attempt to understand how cell-cycle machinery responds to various levels of oxygen exposure under *in vivo* conditions. Exposure of immature lung that is poorly adapted for air breathing to higher levels of oxygen would pose an acute oxidative insult. Even if PRN exposure in the range of 25–50% oxygen is used for mechanical ventilation of immature lung, it will be a se-

TABLE 1. SUMMARY OF RESULTS SHOWN IN FIGS. 1–9

	125-day animals exposed to PRN oxygen			*140-day animals exposed to PRN or 100% oxygen*		
	GC	*6 days of PRN O_2*	*14 days of PRN O_2*	*GC*	*6 days of PRN O_2*	*6 days of 100% O_2*
Cyclin D1	+	++	+++	+	++	++
Cyclin E	+	+++	++	++	+++	−
Cyclin A	+++	++	++	+++	++	+
Cdk4	+	+++	++	+	+++	+
Rb	+++	++	+	+++	++	+
Phospho-Rb	+	+++	++	+	++	+
Cyclin B1	+	+++	++	+	+++	−
$p34^{cdc2}$	+++	+++	++	+++	+++	−
Phospho-$p34^{cdc2}$	+	+++	+	++	++	+

vere oxidative insult to the developing lung that lacks critical antioxidant enzymes to adapt itself for air breathing.

Lung development requires an ordered pattern of proliferation and differentiation of various lung cells that culminates in the development of alveolus, airways, and various specialized cells that are required for air breathing. Therefore, normal cell-cycle progression is crucial for lung development. Orderly progression of the cell cycle requires cyclic appearance and degradation of cyclins that are positive regulatory components of cdks whose activation is required for progression through G1, S, and M phases of the cell cycle. As hyperoxia has been shown to modulate various cyclins and cdks in cell culture models, we examined whether the expression of cyclins is modulated in BPD, which is an oxidative lung disease.

The level of G1 cyclins, such as cyclin D1, cyclin E, and cyclin A, increases in G1/S phase, which recruits cdk4 or cdk2, and the association results in active cyclin-cdk complex. Cyclin D-cdk4 or cyclin E-cdk2 phosphorylates Rb at multiple sites that dissociate E2F transcription factor, which activates various genes required for DNA replication. Our observation that cyclin D1 expression increased in 125-day or 140-day animals exposed to oxygen suggests that oxygen induces the expression of this protein. Cyclin D1 and D2 have been shown to be up-regulated in rat epithelial type II cells exposed to 95% oxygen for 24 or 48 h (10). However, the activity of cyclin D-cdk4 has been shown to be decreased in hyperoxia in the same cell culture model (10). We have also observed that cyclin E expression increased after 6 days of PRN exposure in 125-day or in 140-day premature infants. However, prolonged exposure of 125-day animals (14 days of PRN) or exposure to 6 days of 100% oxygen in 140-day animals demonstrated decreased cyclin E expression. Thus, it appears that 125-day animals exposed for 6 days to PRN oxygen may be undergoing repair by increasing the proliferation of cells, but prolonged exposure may impair the expression of cyclins, resulting in failure of repair of the damaged alveolar epithelium or bronchiolar epithelium. Alternatively, the severity of injury could have been decreased at the end of 14 days of PRN exposure due to less FiO_2 (inspired oxygen fraction) (24).

The 140-day animals exposed to 100% oxygen for 6 days demonstrated decreased expression of cyclins and cdk4 expression. Exposure to 100% oxygen for 6 days may pose an acute oxidative stress in the poorly adapted immature lung. The severity of lesion in these animals is characterized by epithelial loss, hyaline membrane/saccular edema, interstitial edema, and endothelial edema or necrosis. Thus, decreased expression of cyclins and cdks in this model may cause failure of epithelial repair and, therefore, accelerated progression of initial injury.

The expression and activity of the G1 cyclin-cdk complex phosphorylate Rb protein, which is required for the progression of the cell cycle. Rb protein remains hypophosphorylated in resting cells whose level is relatively constant throughout the cell cycle. We observed decreased expression of Rb protein in 125-day animals exposed to 6 or 14 days of PRN oxygen exposure. Additionally, the phosphorylation of Rb increased in 125-day animals exposed for 6 and 14 days to oxygen. In contrast, Rb phosphorylation decreased in animals exposed to 6 days of 100% oxygen. These studies indicate that Rb protein may be a limiting factor in BPD that may arrest the cell cycle. Furthermore, 140-day premature lung subsequently exposed to 6 days of 100% oxygen may undergo cell-cycle arrest due to both decreased Rb level and phosphorylation.

Lung cells have been shown to be arrested at the G2/M phase boundary in response to hyperoxia (22). However, whether G2 arrest occurs in BPD is yet to be determined. Cyclin B1 and $p34^{cdc2}$ are the major regulatory cyclin and cdk of the G2/M phase transition. In the late G2 phase, cyclin B1 expression increases, which binds to $p34^{cdc2}$, resulting in the activation of kinase activity that is required for phosphorylation of various substrates in the M phase of the cell cycle (19). We observed increased expression of cyclin B1 in 125-day animals exposed to 6 and 14 days of PRN oxygen. However, the expression of cyclin B1 was decreased in 140-day animals exposed to 100% oxygen. Cyclin B1 expression has been shown to be decreased in cultured lung cells exposed to 95% oxygen (22). Although cyclin B1 expression increased in 125-day animals, the phosphorylation of $p34^{cdc2}$ increased in these animals after 6 or 14 days of PRN oxygen exposure. As phosphorylation of $p34^{cdc2}$ inactivates the kinase activity of the cyclin B1-$p34^{cdc2}$ complex, lung cells may be undergoing a G2 arrest in BPD.

Thus, the data obtained in this study suggest that lung cells may be undergoing G1 as well as G2 arrest in premature in-

fant baboons in BPD. We have recently demonstrated that p21 and p53 expression increases in the premature infant baboon model of BPD. p21 is a cdk inhibitor, which inhibits the cdk activity by binding to G1 cyclin-cdk complexes such as cyclin D-cdk4 or cyclin E-cdk2. Thus, it could be possible that increased p21 expression may inhibit the cyclin D-cdk4 complex in BPD. However, further studies may be required to evaluate these possibilities.

A recent study of BPD in the baboon model noted increased proliferation of pro-surfactant protein B (pro-SP-B) secreting cells (20). Although this study recorded a higher rate of proliferation of pro-SP-B cells for 125-day baboons in CLD, the proliferation of other cell types is yet to be determined. Additionally, the nuclear antigen Ki67 may not fully account for all the proliferating cells. Cells can be Ki67-positive, yet they may be arrested in certain phases of the cell cycle (26). We observed increased p21 and p53 expression in 125-day or 140-day baboons with BPD (11), which suggests that growth arrest may occur in these affected infants. In addition, we found decreased expression of Rb in 125-day or 140-day premature animals that were exposed to 6 days of PRN or 100% oxygen, which suggests a possible G1 arrest of lung cells in BPD. However, further studies may be required to establish conclusively the role of cyclins and cdks in the evolution of BPD.

Baboons at 125 days or 140 days of gestation were used in this study (term = 185 days). The histopathology of animals at this stage of prematurity has been well characterized (6, 7). The differentiation of alveolar cells to type I or type II has not been observed in 125-day animals. Therefore, in our study (125-day animals), it was not possible to determine whether type II cells express more proliferative response to repopulate damaged type I cells. However, further studies with specific markers for various cell types may answer this question. Our present study demonstrates that cell-cycle regulatory proteins are modulated in hyperoxia, which may, in part, account for the disordered proliferation, a characteristic of BPD.

Although this study presents data on the expression of important cell-cycle regulatory cyclins and cdks in CLD, it has several limitations. First, the number of animals that underwent premature delivery and oxygen ventilation was relatively small (three to four animals per group). Second, the effect of mechanical ventilation with various concentrations of inspired oxygen may not have been identical for each animal, and therefore, its effect on the cyclin or cdk expression may be variable. Third, the effect of various nutrients, surfactants and antibiotics on the expression of cyclins and cdks is unknown.

In summary, the present study demonstrated that the expression of various cell-cycle regulatory proteins is modulated in CLD of premature and extremely premature primates. Modulation of these proteins may set the stage for disordered lung architecture and development in CLD.

ACKNOWLEDGMENTS

The authors acknowledge the superb and dedicated assistance of numerous physicians, scientists, nurses, technicians, and other personnel at the BPD Resource Center in San Antonio. The authors are thankful to Dr. J.J. Coalson and Vicki Winter for providing the paraffin-embedded sections. Dr. Das was supported by a Scientist Development Award from the American Heart Association National Center and a research project grant from the American Cancer Society.

ABBREVIATIONS

BPD, bronchopulmonary dysplasia; CLD, chronic lung disease; GC, gestational control; MPF, maturation promoting factor; p21, Waf1-cyclin kinase inhibitor 1A; $p34^{cdc2}$, 34-kDa cyclin-dependent kinase-1; p53, tumor protein p53; paO_2, partial pressure of oxygen; PRN, pro re nata (as occasion requires); pro-SP-B, pro-surfactant protein B; Rb, retinoblastoma protein.

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