Dendritic-like Cells Accumulate in BPD Lung of Baboon

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The present investigation demonstrates HLA-DR and PGP9.5 double positive cells accumulate thymus cortical region in normal baboon thymus and baboon lung. But, these cells disappeared in thymus and lung of bronchopulmonary dysplasia (BPD) animals. 125d GC animal model is more suitable for BPD than 140d GC animal. Anti-bombesin antibody, 2A11 treated baboon recover normal level of HLA-DR positive cells from BPD animal. In addition, thymocytes show responsiveness for bombesin. These observation suggest that blocking BLPs protects a chronic lung injury by BPD and 2A11 is possible agent for passive therapy of BPD.

Key words : Bronchopulmonary dysplasia (BPD), 2A11, *pro re nada* (PRN), baboon, autoreactivity

Introduction

Bronchopulmonary dysplasia (BPD) is a chronic lung disease of premature infants with multiple causes including oxygen toxicity, barotrauma, infection, and lung immaturity [10-11]. This disease leads to increased morbidity and mortality [1] compounding severity in premature newborns. Although modern techniques have been developed to reduce its severity, BPD still have the clear mechanistic pathways to be elucidate or methods for the potent care therapy. To demonstrate BPD mechanism *in vivo* system, there are two baboon model established by Escobedo [8] and Coalson [4,5]. First is the hyperoxic model. That is, preterm baboons delivered at 140d gestation (full term=185 days) are cultivated in chamber supplied with 100% O₂ for 10d and develop severe BPD. However, 140d animals given maintenance levels of O₂ *pro re nada* (PRN) do not develop any clinical and pathologic BPD changes. Recently, Coalson have developed a more mild BPD model with 125d gestation preterm baboon ventilated O₂ *pro re nata* (PRN) (to keep arterial blood hemoglobin above approximately 90% O₂ saturation) (125d/PRN) characterized as alveolarization.

Bombesin identified by Vittorio Ersparmer from frog *bombina bombina* skin [7] was a small peptide composed of 14 amino acid. Bombesin homologue, bombesin-like peptide (BLP) termed as gastrin-releasing peptide (GRP) [12]

was detected with amphibian bombesin antibody. BLP can facilitate the organogenesis of fetal lung in humans, non-human primates, rats, and mice [15-17]. Immuno-reactivity for BLP is widely found in the central nervous system, the gut, and the lung, but the highest immuno-reactivity is detected in mid-gestation human fetal lung. While a lung organogenesis is processing, the first lung epithelial cells to differentiate are the pulmonary neuroendocrine cells [6,18] which show high levels of BLP immunoreactivity. Increased numbers of BLP-positive pulmonary neuroendocrine cells (PNEC) lead in newborns with BPD. In addition, thymus involution is excellent in infants with BPD.

In this report, we address cell types involved in BPD between normal thymus, lung and BPD thymus, lung. 2A11 antibody for BLP verifies the possibility of passive therapy for BPD.

Materials and Methods

Animal

All animal studies were performed according to the National Research Council's Guide for the Care and Use of Laboratory Animals. Normal fetal baboons were delivered by Caesarean section at 125d and 145d gestation. Lung and thymus tissue were harvested aseptically, flash frozen and stored at -70°C. Premature baboons delivered at 125d were administered anti-bombesin antibody 2A11 intravenously at 5 mg/kg beginning 2h after birth, followed by 2 mg/kg on Day 3, 6, and 9. 125d gestation preterm baboons venti-

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lated for 14d and 21d on oxygen *pro re nata* (PRN) (to keep arterial blood hemoglobin above approximately 90% O₂ saturation). Details of the protocol have been described previously [12].

Immunostaining

Thymus and lung tissues were fixed in 4% paraformaldehyde, and paraffin-embedded [14]. Immunostaining was used after antigen retrieval with Ab-972 (Abcam, Cambridge, MA). Antibodies were as follows: Rabbit anti-protein gene product 9.5 (PGP9.5) (Utracclone, Cambridge, UK) at 1:100 and HLA-DR (1:100 mouse anti-human HLA-DR; BD Biosciences, San Diego, CA) were incubated for 1hr at 20°C, followed by secondary antibodies for 1hr using Alex Fluor 568 goat anti-rabbit IgG (Molecular Probes, Eugene, OR) and Alex Fluor 488 goat anti-mouse IgG (Invitrogen, Carlsbad, CA). DAPI (300 nM) was used to label nuclei.

Bombesin chemotaxis

Single-cell suspensions were prepared from fresh baboon thymus by passing cells through a fine stainless steel screen into DMEM/5% FBS then through a 200-micropore polyester screen to remove debris. Cell pellets were suspended in DMEM/5% FBS. Chemotaxis assay of thymocytes of normal and BPD animal thymocytes was performed in transwell cell culture chambers with polycarbonate filters, 6.5 mm diameter, 10 µm thickness and 5 µm pore size (Costar). A total of 2×10^5 cells suspended in 100 µl DMEM were added to the upper chamber and 600 µl of the same medium were added to the lower well. When indicated, 10 nM and 100 nM bombesin were added to the lower well. Cells were allowed to migrate for 2 hr at 37°C and 5% CO₂ incubator. Counting of migrated cells was carried out using a FACScalibur flow cytometer using Cell Quester software (BD Biosciences). The assay was carried out at least in duplicates and the results were expressed as the average.

Results and Discussion

HLA-DR positive-thymic nurse cells (TNC) are accumulated in baboon thymus cortical region.

To investigate thymic cortical epithelial nurse cells in thymus of preterm baboon (140GC), we used HLA-DR immunofluorescence staining for thymic nurse cell detection

Fig. 1. HLA-DR positive cells show dendritic-like cell morphological feature in high magnification of 140d GC of baboon thymus tissue. Thymus tissues were fixed in 4% paraformaldehyde, and paraffin-embedded. Immunostaining was used after antigen retrieval with Ab-972. HLA-DR (1:100) were incubated for 1 hr at 20°C, followed by Alex Fluor 488 goat anti-mouse IgG. DAPI (300 nM) was used to label nuclei. M: medulla, C: cortex

of thymus [2]. Surprisingly, we detected that large numbers of HLA-DR positive dendritic like cells are accumulated in thymus cortical region of 140d gestation preterm baboon (Fig. 1). However, rare numbers of HLA-DR positive cells are detected in medulla region (Fig. 1). In pre-term baboon, it seems that HLA-DR positive cells play a crucial role for immature lymphocyte selection.

Thymic nurse cells accumulated in thymus cortical region are double positive cells for HLA-DR and PGP9.5.

The involution of thymus occurs either as a normal development stage during physiologic process or as a pathologic process in premature neonates with acute injury, infection. To verify the identity of the HLA-DR positive cells as thymic nurse cell, we carried out double staining with 140d gestation control and 125d 21d/PRN thymus of baboons for HLA-DR and PGP9.5 [3]. In 140d GC, numerous cells are observed in the cortical region with double positive signal (Fig. 2). However, HLA-DR and PGP9.5 double positive cells are disappeared in cortical region of 125d 21d/PRN animal (Fig. 2). Numerous thymic nurse cells and thymic dendritic cells were observed in normal full-term infant baboons in spite of general physiologic thymic involution [19]. Thymic nurse cells in 125d/PRN

Fig. 2. HLA-DR positive cells are positive for PGP9.5 antigen in baboon thymus tissues. Thymus were fixed in 4% paraformaldehyde, and paraffin-embedded. Immunostaining was used after antigen retrieval with Ab-972. Rabbit anti-protein gene product 9.5 (PGP9.5) at 1:100 were incubated for 1 hr at 20°C, followed by secondary antibodies for 1hr using Alex Fluor 568 goat anti-rabbit IgG. DAPI (300 nM) was used to label nuclei. M: medulla, C: cortex

baboons are reduced and this appears to be involved in a pathologic condition, suggesting that thymic nurse cells do not survive during involution process associated with pathologic event. BPD animals show increased autoimmune reactivity [9,13]. Possible mechanism may be involved in the reduction of thymic nurse cells that can regulate thymocyte development, differentiation and positive-negative thymocyte selection.

Normal 125d GC and 140 GC animals show HLA-DR positive-signal in baboon lung tissues.

To induce mild BPD baboon, we used 125d preterm baboon. Lung sections from 125d GC and 140d GC baboon were immunostained for HLA-DR. First, we detected positive signal from lung tissue for HLA-DR throughout the interstitial lung parenchyma of 125d GC and 140d GC preterm baboon lung (Fig. 3). In addition, more HLA-DR positive cells with a dendritic morphology are observed in 125d GC than 140 GC baboon lung (Fig. 3). Thus, 125d GC animal is better than 140GC animal for BPD animal model, taking advantage of time saving and easy-handle.

HLA-DR positive dendritic-like cells are downregulated in lung with BPD.

BLP can be produced by pulmonary neuroendocrine (NE) cell and thymic NE cells [14]. Especially, the expression level of BLP is elevated in BPD animals. Anti-BLP blocking antibody 2A11 treatment abrogates thymic cortical involution in BPD animals [14]. Therefore, BLP is one of target molecules in passive therapies. To address the

Fig. 3. Normal 125d GC and 140GC long feature the accumulation of HLA-DR positive cells. Lung tissues were fixed in 4% paraformaldehyde, and paraffin-embedded. Immunostaining was used after antigen retrieval with Ab-972. HLA-DR (1:100) were incubated for 1 hr at 20°C, followed by secondary antibodies for 1 hr using Alex Fluor 488 goat anti-mouse IgG. DAPI was used to label nuclei.

ability of 2A11 against BLP, lung tissues of 2A11 treated animal were carried out with immunostaining for HLA-DR. HLA-DR positive cells were detected from 140d GC and 125d014d 2A11 treated animal (Fig. 4). In contrast, 125d-14d/PRN and 125d-21d/PRN animal showed rare HLA-DR positive cells from lung interstitium (Fig. 4). It suggests that BLP can down-regulate pulmonary dendritic-like cells in BPD lung tissue. Thus, 2A11 may play a potent immunomodulatory role reducing anti-lung auto-reactivity or as a passive therapy.

Fig. 4. Downregulation of dendritic cells in lung with BPD. Normal 140d GC, 125d-14d 2A11 treated baboon long feature the accumulation of HLA-DR positive cells, however, 125d-14d/PRN and 125d21d/PRN baboon long show rare HLA-DR positive cells. Lung tissues were fixed in 4% paraformaldehyde, and paraffin-embedded. Immunostaining was used after antigen retrieval with Ab-972. HLA-DR (1:100) were incubated for 1 hr at 20°C, followed by secondary antibodies for 1 hr using Alex Fluor 488 goat anti-mouse IgG. DAPI was used to label nuclei.

Thymocytes from normal 140d GC animal thymus response for bombesin peptide

BPD occurs by multi-factory causes. Typically, thymus involution is observed in premature infants of BPD animals. Thymus involution by an abnormal process such as BPD have an influence of positive-negative selection of premature thymocytes in thymus. In BPD baboons, proliferation of premature thymocytes was detected in thymic cortical region [14]. In order to observe a responsiveness of thymocytes for bombesin peptide, we performed chemotaxis assay using transwell *in vitro* system. Thymocytes of 140d GC thymus show high migratory responsiveness for bombesin (Fig. 5). It seems that proliferated premature thymocytes emit from thymus without positive-negative selection toward BPD lung which express bombesin-like peptide of high level by PNE cells. It suggests that one candidate mechanism of BPD is auto-immune reactivity attacking self-lung tissue by premature thymocytes not carrying out selection blocking BLPs can protect a chronic lung injury occurred by BPD.

Fig. 5. Thymocytes from normal 140d GC animal thymus migrate toward bombesin peptide. Single-cell suspensions were prepared from fresh baboon thymus by passing cells through a fine stainless steel screen into DMEM/5% FBS then through a 200-micropore polyester screen to remove debris. Cell pellets were suspended in DMEM/5% FBS. Chemotaxis assay for normal 140d GC thymocytes was performed in transwell cell culture chambers with polycarbonate filters, 6.5 mm diameter, 10 μ m thickness and 5 μ m pore size. Counting of migrated cells was carried out using a FACScalibur flow cytometer using Cell Quester software. The assay was carried out at least in duplicates and the results were expressed as the average.

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초록 : 만성 폐질환인 폐이형성증을 보이는 baboon 폐조직에 Dendritic-like cell의 축적

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본 연구에서 정상적인 baboon의 흉선조직 및 폐조직에서 HLA-DR과 PGP9.5항원에 대한 반응성을 나타내는 수지상세포 형태의 세포가 축적되는 것을 보았다. 하지만, 이런 세포들은 baboon 이 BPD즉 폐이형성증에 걸리게 되면 사라진다는 것을 확인하였다. 그리고 140일 제태기간보다 125일 제태 기간을 보낸 미성숙 baboon이 HLA-DR을 가지는 세포가 더 많아 진다는 것을 관찰할 수 있었고 이로 인해 BPD동물모델을 구축하는데 125일 제태 기간을 보낸 동물이 훨씬 적합하다는 것을 알았다. 또한 bombesin의 항체인 2A11을 처리한 baboon (125d-14d/PRN)의 폐조직에서는 정상수준의 HLA-DR positive세포들이 관찰되었다. 더욱이, Baboon의 흉선으로부터 분리한 thymocyte로 bombesin에 대한 chemotaxis를 수행한 결과 세포이동 반응성을 보였다. 이러한 결과들은 생체 내 BLP들을 차단하면 BPD에 의해 야기된 폐이형성증을 막을 수 있고 2A11항체가 수동 면역제로 폐이형성증을 치료할 수 있는 치료제로써의 가능성을 제시하는 것이다.