Bone marrow-derived cells contribute to lung regeneration after elastase-induced pulmonary emphysema

Kota Ishizawa[a], Hiroshi Kubo[a,*], Mitsuhiro Yamada[a], Seiichi Kobayashi[a], Muneo Numasaki[a], Shinsaku Ueda[b], Takashi Suzuki[c], Hidetada Sasaki[a]

[a] *Department of Geriatric and Respiratory Medicine, Tohoku University School of Medicine, Sendai, Miyagi 980-8574, Japan*
[b] *Miyagi Cancer Centre, Natori 989-1239, Japan*
[c] *Department of Pathology, Tohoku University School of Medicine, Sendai, Miyagi 980-8574, Japan*

Received 21 September 2003; revised 13 November 2003; accepted 13 November 2003

First published online 12 December 2003

Edited by Veli-Pekka Lehto

Abstract All-*trans* retinoic acid (ATRA) is known to reverse the anatomic and physiologic signs of pulmonary emphysema. However, the origin of the progenitor cells involved in this lung regeneration remains unclear. Recently, it was shown that bone marrow could be the source of progenitor cells for several cell types. Mice with elastase-induced emphysema were treated with ATRA, granulocyte colony-stimulating factor (G-CSF), or a combination of both. ATRA or G-CSF promoted lung regeneration and increased bone marrow-derived cell (BMC) numbers in alveoli. Combined treatment of both had an additive effect, which indicated that BMC mobilization might be important in lung regeneration.

Key words: Bone marrow; Progenitor cell; Pulmonary emphysema; Granulocyte colony-stimulating factor; Regeneration

1. Introduction

Pulmonary emphysema is one of the most common reasons for ill health worldwide, and is ranked as a frequent cause of death. The pathophysiologic changes associated with emphysema are the progressive destruction of lung parenchyma and reduced surface area available for gas exchange, which result in inadequate oxygenation. Because adult human lungs cannot spontaneously grow or regenerate, pulmonary emphysema is considered to be a progressive and irreversible disease. In 1997, Massaro et al. showed that all-*trans* retinoic acid (ATRA) reversed anatomic and physiologic signs in an animal pulmonary emphysema model [1]. ATRA is known to activate genes involved in lung development and promote alveolar septation and lung growth. However, the origin of the progenitor cells that regenerated the damaged lung parenchyma remains unclear.

It has recently been shown that the bone marrow is the source of progenitor cells for various cell types, including endothelial cells [2], epithelial cells [3–5], myocytes [6], and neurons [7]. Also, the mobilization of bone marrow-derived cells (BMCs) by cytokines, such as granulocyte colony-stimulating factor (G-CSF), has been shown to be involved in the repair of the infarcted heart [8]. Therefore, we hypothesized that BMCs may contribute to lung regeneration in the emphysematous lung. The aims of this study were to examine the effect of hematopoietic cell mobilization in lung regeneration, and to evaluate the distribution of BMCs after regeneration.

2. Materials and methods

2.1. Lethal irradiation and reconstitution of bone marrow

Mice transgenic for enhanced green fluorescent protein (GFP) on a C57BL/6 strain background were established at the Research Institute for Microbial Disease, Osaka University, Japan [9]. Adoptive transfer of fetal liver cells was performed according to a previously described method [10]. Briefly, embryos were generated from crosses of GFP mice. Fetal livers were harvested from day 13.5 GFP embryos (where day 0.5 is defined as the morning the plugs were identified) and placed in BMC media (Hank's balanced salt solution, 10 mM HEPES, 2.5 fetal bovine serum). Single-cell suspensions were prepared by passage through a 26-gauge needle. Recipient C57BL/6 male mice were irradiated using doses of 8 Gy and 4 Gy, separated by 3 h. Bone marrow was reconstituted by injecting fetal liver cells (2×10^6 cells/200 μl media) intravenously through the tail vein. Three weeks after transplantation, over 95% of the circulating white blood cells were GFP-positive, indicating that the recipient C57BL/6 mice had completely reconstituted with BMCs of GFP mouse origin.

2.2. Induction of pulmonary emphysema and treatment

After confirming GFP chimerism, lung emphysema was induced in mice by intranasal instillation of porcine pancreas elastase (200 units/kg; Sigma, St Louis, MO, USA), as previously described [1]. Three weeks after elastase administration, mice exhibited emphysematous changes in the lungs. At this point, mice were randomly divided into four groups (each group $n=5$) and were administered either vehicle only, ATRA (500 μg/kg/day, intraperitoneally (i.p.); Sigma), G-CSF, a factor known to mobilize hematopoietic and mesenchymal lineage cells from bone marrow [11] (50 μg/kg/day, subcutaneously (s.c.); PeproTech, Rocky Hill, NJ, USA), or a combination of both ATRA and G-CSF. All reagents were administered by daily injection for 12 days, after which the mice were killed for analysis.

2.3. Histological analysis of recipient lungs

Pulmonary vasculature was flushed with phosphate-buffered saline (PBS) to wash out the intravascular leukocytes, because leukocytes remaining (most of them were GFP-positive) within the pulmonary capillary disturb the histological evaluations. To flush the pulmonary vasculature, the right ventricle was punctured with a 23-gauge needle (TERUMO, Tokyo, Japan). The pulmonary vessels were perfused in situ with PBS at a pressure of 20 cmH_2O. Then, recipient lungs were fixed with 4% paraformaldehyde–PBS at a transpulmonary pressure

*Corresponding author. Fax: (81)-22-717 7186.
E-mail address: hkubo@geriat.med.tohoku.ac.jp (H. Kubo).

Abbreviations: ATRA, all-*trans* retinoic acid; BMC, bone marrow-derived cell; G-CSF, granulocyte colony-stimulating factor; GFP, green fluorescent protein; Sca-1, stem cell antigen-1; Flk-1, fetal liver kinase-1; Lm, mean linear intercept

0014-5793/03/$30.00
doi:10.1016/S0014-5793(03)01399-1

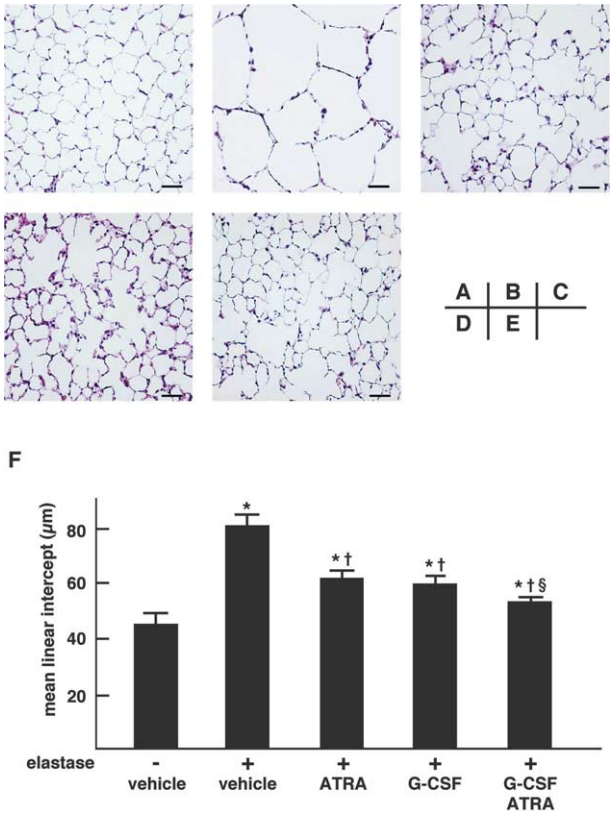

Fig. 1. ATRA and G-CSF reduced the extent of emphysematous changes. A–E: Representative histological sections for each group. Sections were stained with hematoxylin and eosin. Elastase treatment induced emphysematous changes in murine lungs (B), compared to non-treated lungs (A). Treatment with ATRA (C), G-CSF (D), or both ATRA and G-CSF (E) increased alveolar septation and reversed the manifestations of elastase-induced emphysema. F: The alveolar Lm, used as a morphometric parameter of emphysema, showed that treatment with ATRA, G-CSF, or ATRA+G-CSF reduced emphysematous changes. $^{*}P < 0.001$, versus vehicle without elastase, $^{\dagger}P < 0.001$ versus vehicle with elastase, $^{\S}P < 0.001$ versus ATRA.

of 20 cmH_2O. Fixed lungs were embedded in paraffin and sectioned using standard methods. Fixed lungs were also washed in sucrose–PBS, embedded in ornithine carbamoyltransferase (OCT) compound, and frozen in liquid nitrogen for cryosectioning. Paraffin sections were processed for hematoxylin and eosin staining or for immunohistochemistry. GFP was detected using rabbit polyclonal anti-GFP antibody (Abcam, Cambridge, UK), with horseradish peroxidase (HRP)-labelled goat anti-rabbit IgG (Vector, Burlingame, CA, USA) used as the secondary antibody. Samples were subsequently incubated with avidin-biotin HRP complex (ABC Elite, Vector). The final reaction product was visualized with 3,3′-diaminobenzidine (Sigma). Sections were counterstained with hematoxylin. The extent of the emphysematous lesions was assessed by measuring the mean linear intercept (Lm) using the method of Thurlbeck with some modifications [12]. Briefly, 20 fields at 400× magnification were randomly sampled on two slides from each mouse, and point counting performed. The total distance divided by the number of alveolar intercepts gave Lm. Cryosections (10 μm thick) were processed for immunofluorescent staining. Alveolar epithelial cells were identified using a murine monoclonal antibody anti-cytokeratin 5 and 8 (Chemicon, Temecula, CA, USA) and a goat Alexa fluor 350-conjugated anti-mouse antibody (Molecular Probes, Eugene, OR, USA). Endothelial cells were identified using a rat biotin-conjugated monoclonal anti-murine CD34 antibody (BD Pharmingen, San Diego, CA, USA) and 7-amino-4-methylcoumarin-3-acetic acid (AMCA)-conjugated streptavidin (Immunotech, Marseille, France). Hematopoietic cells were identified using a rat phycoerythrin (PE)-conjugated anti-murine CD45 antibody (BD Pharmingen). The number of GFP-positive and CD45-negative cells, consistent with BMCs excluding intravascular or perivascular hematopoietic cells, was counted in alveolar walls of 200 separate alveoli from each mouse (each group $n = 5$). Histological evaluations were performed blindly by three observers (K. Ishizawa, T. Suzuki, and H. Kubo). In the present study, inter-observer differences were less than 5%, and the mean of the three values was obtained.

2.4. Cell sorting and flow cytometry

A total of 0.5–1.0 ml of blood was obtained from each mouse. Red blood cells were depleted using RBC lysis buffer (0.15 M NH_4Cl, 0.01 M $KHCO_3$, 0.1 mM ethylenediamine tetraacetic acid (EDTA)-2Na, pH 7.2). Stem cell antigen-1 $(Sca\text{-}1)^+$ peripheral blood cells were isolated using the Magnetic Cell Sorting System with anti-Sca-1-coated microbeads (Miltenvi Biotec, Bergisch Gladbach, Germany). Sorted $Sca\text{-}1^+$ cells were stained with fluorescein isothiocyanate (FITC)-labelled anti-murine Sca-1 antibody (BD Pharmingen) and PE-labelled anti-murine fetal liver kinase-1 (Flk-1) (BD Pharmingen), and analyzed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Cells were initially gated by propidium iodine (Sigma, St. Louis, MO, USA) staining to restrict consideration to live cells.

2.5. Statistical analysis

Data are expressed as the mean ± standard error of the mean. Comparisons were made by analyses of variance, and when overall differences were identified, multiple contrasts with a Bonferroni adjustment were used to identify which groups were significantly different. Statistical significance was defined as $P < 0.05$.

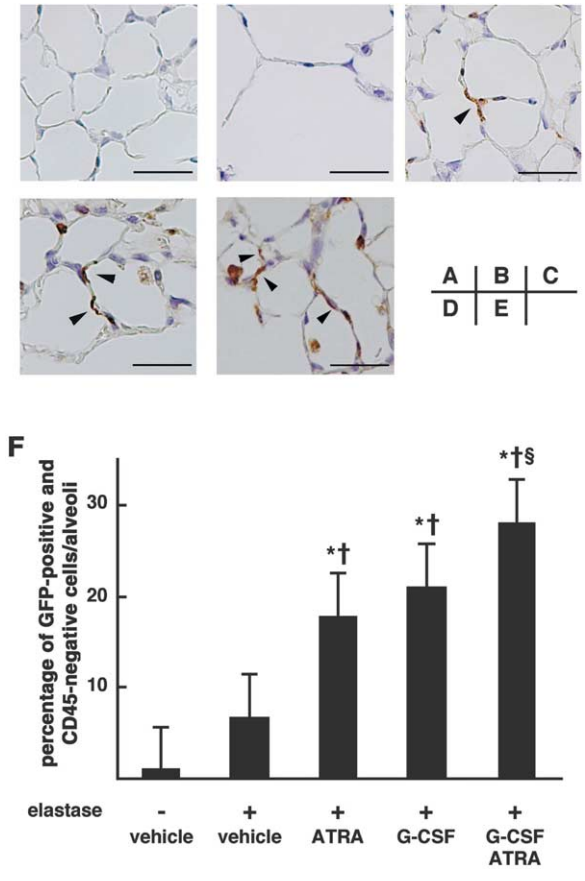

Fig. 2. BMCs are present in regenerated lung parenchyma following elastase-induced emphysema. A–E: Paraffin sections were immunostained for GFP (brown). BMCs $(GFP^+$ cells) are present in the alveolar cell walls (arrowheads). Scale bars, 50 μm. F: Percentage of $GFP^+/CD45^-$ cells in alveoli. $^{*}P < 0.001$, versus vehicle without elastase, $^{\dagger}P < 0.001$ versus vehicle with elastase, $^{\S}P < 0.001$ versus ATRA.

3. Results and discussion

Our results indicated that ATRA treatment improved emphysema, as previously described [1] (Fig. 1A–C). Interestingly, G-CSF treatment alone also provided a significant reduction in emphysema (Fig. 1D). For G-CSF-treated mice, the alveolar Lm, used as a morphometric parameter of emphysema [12], showed a 44% reduction compared to vehicle-treated elastase-induced emphysematous mice (Fig. 1F). This was the same degree of reduction as observed for ATRA-treated mice (Fig. 1F). Combined treatment with G-CSF and ATRA exhibited an additive effect with a 73% reduction in Lm (Fig. 1E,F).

To evaluate BMC distribution, we performed immunohistochemical analysis using an anti-GFP antibody (Fig. 2A–E). At baseline, only circulating white blood cells and alveolar macrophages were GFP-positive (GFP^+), and almost no GFP^+ cells were observed in the alveolar walls (Fig. 2A). While elastase administration induced a slight increase in GFP^+ cells in the alveolar walls (Fig. 2B,F), treatment with G-CSF, ATRA, or both induced a significant increase in the number of alveoli containing GFP^+ cells (Fig. 2C–E). The percentage of GFP^+ cells per alveolus also increased in mice treated with G-CSF, ATRA, or both (Fig. 2F). These results suggested that the regenerated alveoli were comprised of BMCs.

To confirm the cell type of the GFP^+ cells, triple-color immunofluorescent staining was performed using CD45 to mark hematopoietic cells and either cytokeratin to mark epithelial cells [13] or CD34 to mark endothelial cells [14]. Flat GFP^+/cytokeratin$^+$/$CD45^-$ cells were present in the alveolar walls of ATRA- and/or G-CSF-treated lungs (Fig. 3A). Because the population of circulating GFP^+/$CD45^-$ cells in peripheral blood was very low (0.002%), this suggested that BMCs had differentiated into cells with an alveolar epithelial phenotype. Similarly, flat GFP^+/$CD34^+$/$CD45^-$ cells were also observed, which were suggestive of differentiation toward pulmonary capillary endothelial cells (Fig. 3B). Although $CD45^+$ cells were identified in the same lung sections, no cells stained simultaneously for both CD45 and cytokeratin, or for both CD45 and CD34. We also performed simultaneous staining for cytokeratin and CD34. As seen in Fig. 3C, cytokeratin and CD34 antibodies stained separate cell populations. Thus, our data suggested that BMCs play an important role in the regeneration of lung parenchyma.

To evaluate the role of G-CSF on BMC mobilization, we focused on endothelial progenitor cells (EPCs), because they are present in peripheral blood and can differentiate into mature endothelial cells [2]. Bone marrow-derived circulating EPCs express both hematopoietic stem cell and hemangioblast markers, such as Sca-1 and Flk-1/VEGF-R2. Flow cytometry demonstrated that G-CSF treatment induced a three-fold in-

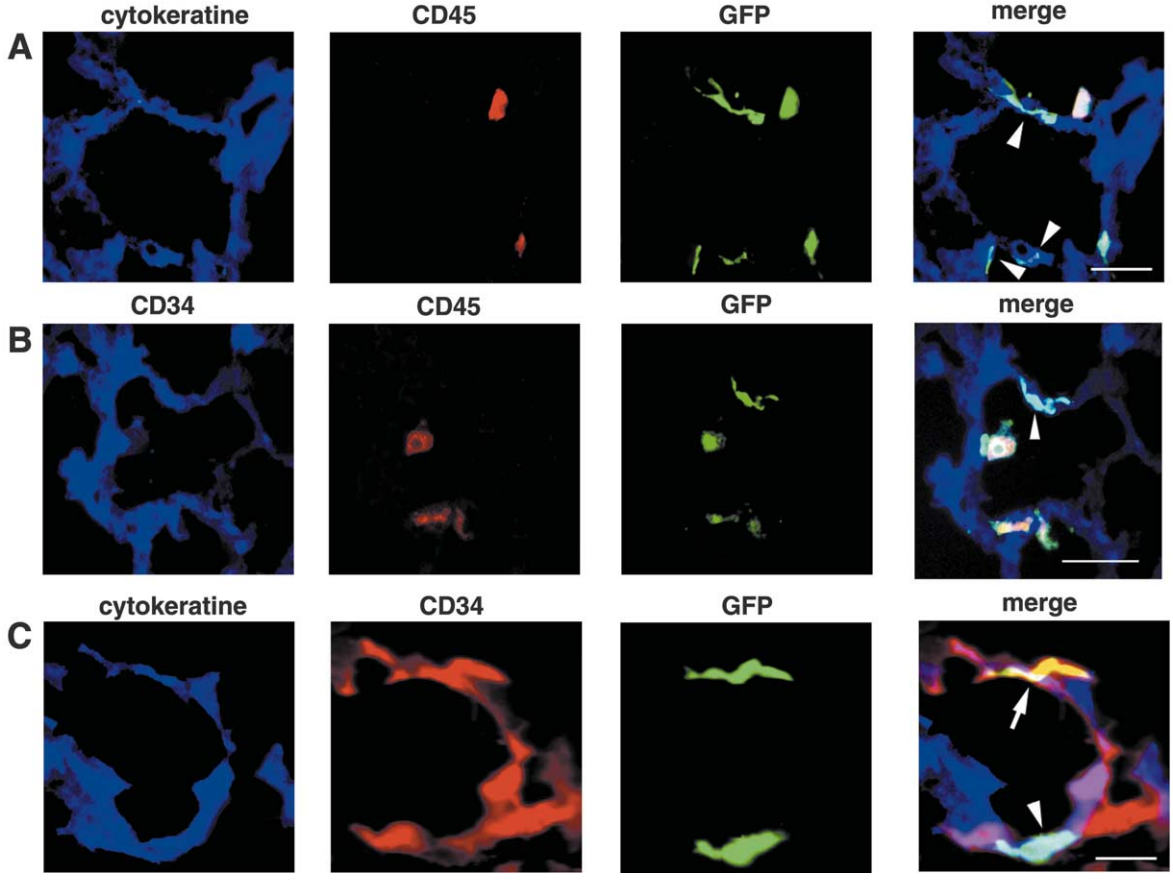

Fig. 3. BMCs differentiate into cells with an alveolar epithelial phenotype or a capillary endothelial phenotype. A–C: Immunostaining for cytokeratin and CD45 (A), CD34 and CD45 (B), or cytokeratin and CD34 (C). Color staining: GFP, green; cytokeratin or CD34, blue; CD45, red (A and B). GFP, green; cytokeratin, blue; CD34, red (C). A and B: Note that GFP^+/cytokeratin$^+$/$CD45^-$ and GFP^+/$CD34^+$/$CD45^-$ cells are present in the alveolar walls (turquoise blue, arrowheads in merged panels). C: Simultaneous staining for cytokeratin and CD34 was also performed. Note that GFP^+/cytokeratin$^-$/$CD34^+$ cell (yellow, arrow in merged panel) and GFP^+/cytokeratin$^+$/$CD34^-$ cell (turquoise blue, arrowheads in merged panel) are present. Scale bars, 10 μm.

crease in the percentage of circulating Sca-1$^+$/Flk-1$^+$ cells in peripheral blood ($0.041 \pm 0.017\%$ in control group versus $0.123 \pm 0.031\%$ in G-CSF group; $n = 7$; $P < 0.001$). In contrast, the percentage of circulating EPCs was not increased by ATRA treatment ($0.058 \pm 0.024\%$; $n = 7$; $P = 0.31$). This suggested that the mobilization of hematopoietic and mesenchymal cells from bone marrow by G-CSF promoted lung regeneration. Also, the roles of ATRA and G-CSF on lung regeneration appear to be different, which would explain why the combined treatment of G-CSF and ATRA had an additive effect. BMCs were present in elastase-treated lungs because of the elastase-induced inflammatory responses (Fig. 2B,F). Although ATRA did not induce EPC mobilization within the circulation, this presence of BMCs may play a role on ATRA-induced lung regeneration and an increase in the number of GFP$^+$/CD45$^-$ cells in ATRA-treated lungs (Fig. 2C,F).

Recently, several studies reported that bone marrow cells adopted the phenotype of other cell types by spontaneous cell fusion rather than by differentiation [15–17]. In our study, the question as to whether GFP$^+$/cytokeratin$^+$/CD45$^-$ alveolar epithelial cells or GFP$^+$/CD34$^+$/CD45$^-$ endothelial cells were generated by BMC differentiation or fusion with existing parenchymal remains unanswered.

While a clinical pilot study of ATRA treatment in patients with pulmonary emphysema has been reported, ATRA treatment failed to show significant improvement in the lung structure as evaluated by CT scan or lung function data [18]. As most of the pulmonary emphysema patients used in the study were elderly, this failure may be due to decreased numbers of progenitor cells in the lung parenchyma or bone marrow. Our present findings may arouse further interest into the therapeutic possibilities of BMCs in the amelioration of pulmonary emphysema.

Acknowledgements: Supported by a grant from the Japan Society for the Promotion of Science 15590792 to H.K. We thank Prof. Masaru Okabe (Genome Information Research Centre, Osaka University, Japan) for providing GFP transgenic mice (C57BL/6 TgN(act-GFP)OsbC14-Y01-FM131).

References

[1] Massaro, G.D. and Massaro, D. (1997) Nat. Med. 3, 675–677.
[2] Asahara, T., Murohara, T., Sullivan, A., Silver, M., van der Zee, R., Li, T., Witzenbichler, B., Schatteman, G. and Isner, J.M. (1997) Science 275, 964–967.
[3] Krause, D.S., Theise, N.D., Collector, M.I., Henegariu, O., Hwang, S., Gardner, R., Neutzel, S. and Sharkis, S.J. (2001) Cell 105, 369–377.
[4] Okamoto, R., Yajima, T., Yamazaki, M., Kanai, T., Mukai, M., Okamoto, S., Ikeda, Y., Hibi, T., Inazawa, J. and Watanabe, M. (2002) Nat. Med. 8, 1011–1017.
[5] Kotton, D.N., Ma, B.Y., Cardoso, W.V., Sanderson, E.A., Summer, R.S., Williams, M.C. and Fine, A. (2001) Development 128, 5181–5188.
[6] Orlic, D., Kajstura, J., Chimenti, S., Jakoniuk, I., Anderson, S.M., Li, B., Pickel, J., McKay, R., Nadal-Ginard, B., Bodine, D.M., Leri, A. and Anversa, P. (2001) Nature 410, 701–705.
[7] Mezey, E., Chandross, K.J., Harta, G., Maki, R.A. and McKercher, S.R. (2000) Science 290, 1779–1782.
[8] Orlic, D., Kajstura, J., Chimenti, S., Limana, F., Jakoniuk, I., Quaini, F., Nadal-Ginard, B., Bodine, D.M., Leri, A. and Anversa, P. (2001) Proc. Natl. Acad. Sci. USA 98, 10344–10349.
[9] Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T. and Nishimune, Y. (1997) FEBS Lett. 407, 313–319.
[10] Horwitz, B.H., Scott, M.L., Cherry, S.R., Bronson, R.T. and Baltimore, D. (1997) Immunity 6, 765–772.
[11] Kocher, A.A., Schuster, M.D., Szabolcs, M.J., Takuma, S., Burkhoff, D., Wang, J., Homma, S., Edwards, N.M. and Itescu, S. (2001) Nat. Med. 7, 430–436.
[12] Thurlbeck, W.M. (1967) Am. Rev. Respir. Dis. 95, 765–773.
[13] Broers, J.L., Carney, D.N., Klein Rot, M., Schaart, G., Lane, E.B., Vooijs, G.P. and Ramaekers, F.C. (1986) J. Cell Sci. 83, 37–60.
[14] Baumheter, S., Singer, M.S., Henzel, W., Hemmerich, S., Renz, M., Rosen, S.D. and Lasky, L.A. (1993) Science 262, 436–438.
[15] Terada, N., Hamazaki, T., Oka, M., Hoki, M., Mastalerz, D.M., Nakano, Y., Meyer, E.M., Morel, L., Petersen, B.E. and Scott, E.W. (2002) Nature 416, 542–545.
[16] Wang, X., Willenbring, H., Akkari, Y., Torimaru, Y., Foster, M., Al-Dhalimy, M., Lagasse, E., Finegold, M., Olson, S. and Grompe, M. (2003) Nature 422, 897–901.
[17] Vassilopoulos, G., Wang, P. and Russell, D. (2003) Nature 422, 901–904.
[18] Mao, J.T., Goldin, J.G., Dermand, J., Ibrahim, G., Brown, M.S., Emerick, A., McNitt-Gray, M.F., Gjertson, D.W., Estrada, F., Tashkin, D.P. and Roth, M.D. (2002) Am. J. Respir. Crit. Care Med. 165, 718–723.