REACTIVE CELL KINETICS IN RAT PULMONARY GRANULOMAS INDUCED BY INTRAVENOUS INJECTION WITH SEPHADEX BEADS

Tomonari Nishimura and Yoshiaki Kawai
Safety Research Laboratory, Tanabe Seiyaku Co., LTD.

Jyoji Yamate and Sadashige Sakuma
Department of Veterinary Pathology, College of Agriculture, University of Osaka Prefecture

Abstract: Macrophages and α-smooth muscle actin immunopositive myofibroblasts are thought to be crucial cells in granuloma formation. In this study, the reactive cell kinetics was investigated in Sephadex bead-induced rat pulmonary granulomas (1 mg/ml saline/head; intravenously) from 1 hour to 90 days after the injection. Intravenously administered Sephadex beads occluded pulmonary arteries and caused granulomatous pulmonary arteritis. The inflammatory cells appeared first around the trapped beads and then granuloma lesions developed within 24 hours. Three days later, the granulomas mainly consisted of neutrophils, macrophages, eosinophils, and giant cells. Immunohistochemically, the number of ED1-positive macrophages quickly increased 24 hours after the injection, and a large number of macrophages were still found 3 days after the injection. Alpha-smooth muscle actin immunopositive myofibroblasts increased in numbers from 3 to 7 days after the injection. These results indicate that the appearance of myofibroblasts in pulmonary granulomas might be mediated by macrophages, which were the most predominant inflammatory cells in the present pulmonary granuloma. (J Toxicol Pathol 9: 29–34, 1996)

Key words: Pulmonary granuloma, Rat, Macrophage, Myofibroblast, Cell kinetics

Introduction

Granuloma is a form of chronic inflammation, and the development of granulomas involves various kinds of inflammatory cells and abnormal accumulation of extracellular matrix material. Recently, mononuclear phagocytes were thought to be the major source of the molecular mediators contributing to granuloma formation and fibrosis after tissue injury1–4. Myofibroblasts are also crucial cells in such lesions, because they produce the extracellular matrix materials leading to fibrosis5–7.

Experimental pulmonary granulomas have also been induced in rats and mice by intratracheal injection of silica8, or by embolization of glucan9, Sephadex beads10,11, and Schistosoma mansoni eggs11. These models have been widely used in order to clarify the mechanism involved in the recruitment, activation, and maintenance of cellular elements in lesions. Sephadex bead-induced pulmonary lesions have been characterized by granulomatous arteritis accompanied by increased eosinophil and macrophage infiltration10. However, the kinetics of macrophage and myofibroblast involvement remains unknown. This study was conducted to clarified reactive cell kinetics in Sephadex bead-induced rat pulmonary granulomas.

Materials and Methods

Animals and Sephadex injection

Thirty-three 7-week-old male F344/DuCrj rats (Charles River Japan) weighing between 163 and 204 g, were used. They were kept in an animal room controlled at 23 ± 1°C and with a 12 hour light-dark cycle. All animals were allowed free access to a standard commercial laboratory diet (CRF-1, Oriental Yeast) and tap water.
Sephadex G-200 beads (Pharmacia Biotech) were administered as previously described\textsuperscript{10,12}. Briefly, autoclaved Sephadex beads were swollen in physiological saline for 48 hours at 4°C, and then they were injected intravenously into the tail vein of 33 rats at a dose of 1 mg/ml/head, under ether anesthesia. Three animals were euthanized at each of the following time points: 1, 4, 8, 12, and 24 hours, and 3, 7, 14, 21, 28, and 90 days after the injection. All animals were intraperitoneally injected with 5-Bromo-2'-deoxy-uridine (BrdU; Sigma) at 100 mg/kg body weight one hour prior to euthanasia.

**Histopathology**

The left lung was fixed in 10% neutral buffered formalin and the other in methyl Carnoy's fixative. Tissues were embedded in paraffin, and 4 μm sections were made. Sections were stained with hematoxylin and eosin (HE), azan stain for collagen fibers, Luna stain for eosinophils, and periodic acid-Schiff reaction (PAS).

**Immunohistochemistry**

Primary antibodies used were as follows: ED1 (1:200) (Serotec) and ED2 (1:100) (Serotec) for rat macrophages/histiocytes, anti-α-smooth muscle actin (α-SMA) antibody (1:100) (Dako) for myofibroblasts, and anti-BrdU antibody (1:200) (Dako) for proliferating cells in the S stage. In the lungs, ED1 recognizes the following; a cytoplasmic antigen of blood monocytes, macrophages in the bronchus-associated lymphoid tissue (BALT), and alveolar macrophages. ED2 reacts with a membrane antigen of tissue macrophages in the perivascular and peribronchial tissues\textsuperscript{13}.

Formalin fixed and deparaffinized tissue sections were used for all staining, except ED2. For the ED2 staining, methyl Carnoy's fixative fixed tissue sections were used. These sections were treated with 0.3% hydrogen peroxide in methanol for 20 minutes to inactivate endogenous peroxidase. After incubation with 5% nonfat dry milk in phosphate buffered saline for 40 minutes, the sections were treated with the above primary antibodies for 120 minutes. Subsequently, they were incubated with biotinylated secondary antibodies (ABC kit; Vector Laboratories) for 30 minutes and with avidin-biotin amplification (ABC kit; Vector Laboratories) for 30 minutes. After visualization with diaminobenzidine (DAB) as a substrate for 5 minutes, the sections were counterstained with hematoxylin. Double immunostaining for ED1 and BrdU was carried out as described\textsuperscript{2}, after DAB visualization (brown reaction) for ED1, DAB nickel-cobalt (Zymed Laboratories) (black reaction) was used as a substrate for BrdU.

ED1-positive macrophages and α-SMA-positive myofibroblasts were counted in 10 randomly selected granulomas from each time point. The mean number per granuloma was calculated.

**Results**

Sephadex bead-induced pulmonary granulomas

At all the time points, Sephadex beads were present in the small caliber muscular pulmonary arteries around the terminal bronchioles. Reactive cell kinetics are shown in Fig. 1. One and 4 hours after the injection, a few neutrophils and macrophages began to aggregate around the Sephadex beads within the arterial lumens and in the vicinity of arteries occluded with Sephadex beads. Eight and 12 hours after the injection, neutrophils and macrophages gradually increased in number. Besides these cells, eosinophils appeared. These inflammatory cells were observed around the trapped beads, within the arterial walls, and in the edematous connective tissues around the affected arteries, giving the appearance of arterial granulomas. Twenty-four hours after the injection, neutrophil numbers were reduced, and they had completely disappeared 3 days after the injection. Sequential observation of granulomatous lesions 24 hours, 3, and 7 days after the injection showed that macrophages and eosinophils (Fig. 3) were the main infiltrators, and that macrophages were much more predominant in the lesions. Only a few ED1-positive macrophages, had a positive reaction to BrdU (Fig. 4) at this stage. In addition, a small number of α-SMA-positive myofibroblasts and multinucleated giant cells were noted. Collagen fibers, stained blue by azan stain, and newly formed vessels were also present. Occasionally cells forming the new vessels were positive for BrdU, indicating the proliferation activity of the endothelia. At later examination points (14 and 21 days), macrophages and eosinophils levels were much lower, and giant cells and...
myofibroblasts were not detected 21 days after the injection. Eosinophils completely disappeared by the 28th day, whereas macrophages were still observed after 90 days. Granulomatous lesions at the 28 and 90 day stages consisted of macrophages, spindle shaped fibroblasts (negative for α-SMA), and small amounts of collagen fibers. BrdU incorporated cells were not observed. Newly formed vessels were still present. At later examination points, Sephadex beads were smaller and had more irregular borders than those at the earlier points (24 hours after injection), indicating partial degradation of the beads.

The kinetics of ED1-positive macrophages and α-SMA-positive myofibroblasts in granulomatous lesions

The appearance of macrophages and the timing of myofibroblast involvement were investigated by

![Graph of cell kinetics](image_url)
counting cell numbers. As shown in Fig. 2, ED1-positive macrophages appeared as early as 1 hour after the Sephadex bead injection, and quickly increased in number up to 24 hours after the injection. Subsequently, the greater number of macrophages were still present after 3 days, having reached a maximal value (Fig. 4). Thereafter, their numbers gradually decreased. However, a small number of macrophages were still observed 90 days after the injection. On the other hand, ED2-positive macrophages were not detected in the granulomatous lesions at any time point.

Alpha-SMA-positive myofibroblasts increased in number and reached a maximum level 3 days after the injection (Fig. 5). At 14, 21, 28, and 90 days, α-SMA-positive cells were not seen.

Discussion

The intravenous injection of Sephadex beads into rats caused peripheral blood eosinophilia and pulmonary arterial granulomas, mainly consisting of eosinophils and macrophages. The development of pulmonary arterial granulomas and the major cell components observed in this study were similar to those reported previously. However, the mechanism of infiltration of various inflammatory cells is not fully understood.

The majority of macrophages responding to tissue injury were thought to be derived from blood monocytes. However, recent studies demonstrated that the increased number of macrophages in cholestatic and toxin-induced liver injury, or partial hepatectomy, in rats depends on both local proliferation and influxes of blood monocytes. In injured lungs, local replication of pulmonary macrophages has been also reported. In this study, a few ED1 and BrdU double-positive macrophages were observed 24 hours and 3 days after the injection, when the number of macrophages had reached a maximum. This finding suggests the local proliferation of macrophages participating in pulmonary granulomas. ED2 specifically recognizes perivascular and peribronchial tissue macrophages. Recruitment of many...
ED2-positive cells into damaged areas has been observed in rats with injured livers. In this study, however, ED2-positive cells were not detected in granulomatous lesions. This may suggest that subpopulations of macrophages recruited into inflammatory lung lesions differ from those in hepatic lesions.

The myofibroblasts have cytoplasmic myofilaments immunoreactive to α-smooth muscle actin, and they have the properties of both fibroblasts and smooth muscle cells. Myofibroblasts are observed in healing skin and experimental fibrosis in the lungs, liver, and kidneys of rats. In addition, myofibroblasts have vimentin, desmin, and actin filaments in their cytoplasm, and their appearance depends on the stage of development and of the myofibroblasts. In the present pulmonary granulomas, increased numbers of myofibroblasts were observed between 3 and 7 days after the injection, followed by development of collagen fibers. The increase was preceded by increases in the number of macrophages. The number of macrophage markedly increased at 24 hours and 3 days after the injection. The appearance of myofibroblasts in these pulmonary granulomas may be mediated by macrophages, as they were the most predominant inflammatory cell type.

In experimental pulmonary, hepatic and renal fibrosis, cell growth factors produced by infiltrating macrophages could transform fibroblasts into myofibroblasts. Chensue et al. showed that macrophages in Sephadex bead-induced mouse pulmonary granulomas produced IL-1 and tumor necrosis factor. Furthermore, transforming growth factor-β1 (TGF-β1) was reported to induce muscle actin expression during bleomycin-induced pulmonary fibrosis. The roles of the myofibroblast in idiopathic pulmonary fibrosis. Ultrasstructural and immunohistochemical features of sites of active extracellular matrix synthesis. Am J Pathol 138: 1257-1265, 1991.


