Experimental and Toxicologic Pathology 68 (2016) 113–124

Contents lists available at ScienceDirect

Experimental and Toxicologic Pathology

journal homepage: www.elsevier.de/etp

Immunophenotypical characterization and influence on liver homeostasis of depleting and repopulating hepatic macrophages in rats injected with clodronate

Munmun Pervin, Hossain M. Golbar, Alexandra Bondoc, Takeshi Izawa, Mitsuru Kuwamura, Jyoji Yamate*

Veterinary Pathology, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-58 Rinku-Ourai-Kita, Izumisano City, Osaka 598-8531, Japan

ARTICLE INFO

Article history:
Received 8 July 2015
Received in revised form 4 November 2015
Accepted 9 November 2015

Keywords:
Kupffer cells
Hepatic macrophages
Immunohistochemistry
Clodronate
Cytokines
Homeostasis

ABSTRACT

Hepatic macrophages (including Kupffer cells) play a crucial role in the homeostasis and act as mediators of inflammatory response in the liver. Hepatic macrophages were depleted in male F344 rats by a single intravenous injection of liposomal clodronate (CLD; 50 mg/kg body weight), and immunophenotypical characteristics of depleting and repopulating macrophages were analyzed by different antibodies specific for macrophages. $CD163^+$ Kupffer cells were almost completely depleted on post-injection (PI) days 1–12. Macrophages reacting to CD68, Iba-1, and Gal-3 were drastically reduced in number on PI day 1 and then recovered gradually until PI day 12. MHC class II^+ and $CD204^+$ macrophages were moderately decreased during the observation period. Although hepatic macrophages detectable by different antibodies were reduced in varying degrees, Kupffer cells were the most susceptible to CLD. Liver situation influenced by depleted hepatic macrophages was also investigated. No marked histological changes were seen in the liver, but the proliferating activity of hepatocytes was significantly increased, supported by changes of gene profiles relating to cell proliferation on microarray analysis on PI day 1; the values of AST and ALT were significantly elevated; macrophage induction/activation factors (such as MCP-1, CSF-1, IL-6 and IL-4) were increased exclusively on PI day 1, whereas anti-inflammatory factors such as IL-10 and TGF-β1 remained significantly decreased after macrophage depletion. The present study confirmed importance of hepatic macrophages in liver homeostasis. The condition of hepatic macrophages should be taken into consideration when chemicals capable of inhibiting macrophage functions are evaluated.

1. Introduction

Showing high plasticity and functional diversity depending on microenvironments, macrophages exist as the resident type in the whole body, and appear as the exudative type in pathological lesions. Liver anchorages the most abundant pool of macrophages in the body of mammals. The representative hepatic macrophages are Kupffer cells (Golbar et al., 2012); additionally, MHC class II-expressing macrophages (as interstitial dendritic cells) are present in the Glisson's sheath in rats (Mori et al., 2009). Hepatic macrophages play a crucial role in the homeostasis and pathogenesis of liver diseases after hepatocytes injury mediated by chemicals. Activated hepatic macrophages after liver injury produce inflammatory/cytotoxic mediators, and anti-inflammatory factors produced by macrophage lead to healing after injury (Laskin 1990; Wijesundera et al., 2014a), indicating biphasic functions of hepatic macrophages. Therefore, recently, macrophages are divided mainly as classically activated inflammatory macrophages (M1) and alternatively activated reparative macrophages (M2) (M1-/M2-macrophage polarization) in rats and mice (Duffield et al., 2005; Martinez et al., 2008; Stein et al., 1992).

To know the functional aspects of macrophages, macrophage depletion has been widely used in normal and pathological conditions (Bautista et al., 2013; Cullen et al., 2013; Sturm et al., 2005). Macrophages can be depleted by injection of liposome-encapsulated dichloromethylene diphosphonate clodronate (CLD) (Van Rooijen and Sanders, 1994). CLD belongs to the family bisphosphonate used for treating osteolytic bone diseases and osteoporosis (Fleisch, 1989). Acting as an anti-macrophage agent, CLD needs to be encapsulated with liposome that is readily digested. Once ingested by macrophages, CLD causes damage to macrophages via apoptosis, resulting in their depletion (Van

* Corresponding author.
E-mail address: yamate@vet.osakafu-u.ac.jp (J. Yamate).

http://dx.doi.org/10.1016/j.etp.2015.11.003
0940-2993/

Rooijen and Sandar, 1994). In the present study, we carried out the detailed immunophenotypical analyses of depleting and repopulating macrophages after CLD injection in rats, using different antibodies specific for rat macrophages. Furthermore, microenvironmental conditions, which may be changed in the absence of hepatic macrophages, were investigated, focusing on hepatic enzymes by biochemical analyses, inflammatory/anti-inflammatory cytokines at mRNA levels and genes relating to homeostasis by comprehensive gene analysis. The data shown in the present study would provide useful information when chemicals with possible hepatotoxicity via hepatic macrophage alterations are evaluated.

2. Materials and methods

2.1. Animals and experimental procedures

Twenty-eight SPF male F344 rats (6 weeks old, 100 to 120 g body weight) were purchased from Charles River Laboratories Japan (Hino, Shiga, Japan), and used after one-week acclimatization. They were housed in an animal room at 21±3 °C, with a 12 h light-dark cycle, and fed a standard diet for rats (DC-8, CLEA Japan, Tokyo, Japan) and supplied with tap water *ad libitum*. Twenty-four rats were given a single injection of liposomal CLD suspension (5 mg/ml in sterile phosphate buffer saline (PBS)) (http://www.clodronateliposomes.org, last accessed on June 17, 2015) at 50 mg/kg body weight intravenously via tail vein; the CLD dose, capable of inducing almost complete depletion of hepatic macrophages after a single injection, was determined based on the preliminary experiment. Along with hepatic macrophages, although the CLD can deplete macrophages in the spleen and bone marrow (Van Rooijen et al., 1990), we focused on immunohistochemical characteristics of hepatic macrophages. Four rats were euthanized by exsanguination under isoflurane anesthesia on each time point of post-injection (PI) days 1, 3, 5, 7, 9, and 12. The remaining four rats were injected an equivalent volume of liposome suspension in PBS, which served as controls and were euthanized on PI day 1. One hour before euthanasia, all rats received intra-peritoneal injection of bromo-2-deoxyuridine (BrdU; Sigma–Aldrich Co., St. Louis, MO, USA) dissolved in physiological saline at 50 mg/kg body weight. The animal experiments were conducted under the institutional guidelines approved by the ethical committee of Osaka Prefecture University for animal care.

2.2. Blood biochemistry

At necropsy, blood was collected from the abdominal aorta, and serum samples were analyzed for aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and γ-glutamyl transferase (γ-GTP) and total bilirubin (T. Bil) by SRL Inc. (Tokyo, Japan).

2.3. Histopathology and immunohistochemistry

Liver tissues from the left lateral lobe were collected and immediately fixed in 10% neutral buffered formalin (NBF), in Zamboni's (0.21% picric acid and 2% paraformaldehyde in 130 mM phosphate buffer, pH 7.4) and in periodate-lysine-paraformaldehyde (PLP) solution processed by PLP-AMeX (acetone, methyl benzoate, and xylene) method (Golbar et al., 2011). NBF-fixed tissues were dehydrated and embedded in paraffin and sectioned at 3–4 μm in thickness. The deparaffinized sections were stained with hematoxylin and eosin (HE) for histopathological examination.

Tissue sections fixed in NBF, Zamboni's solution, or PLP were used for the immunohistochemistry. Deparaffinized tissue sections were used for CD163, CD68, Iba-1, Galectin-3 (Gal-3), MHC class II, CD204 and BrdU staining (Table 1). After pretreatments, tissue sections were stained by the Histostainer (Histofine, Nichirei Bioscience Inc, Tokyo, Japan). Briefly, sections were incubated with 5% skimmed milk in PBS for 10 min, followed by 1 h incubation with primary antibodies. After treatment with 3% H_2O_2 in PBS for 15 min, application of horseradish peroxidase-conjugated secondary antibody (Histofine simple stain MAX PO®; Nichirei Inc., Tokyo, Japan) for 30 min was done. Then, they were incubated with 3,3′-diaminobenzidine (DAB) (Nichirei Inc., Tokyo, Japan) for 5 min. Sections were counterstained with hematoxylin for 1 min. For negative controls, tissue sections were treated with mouse or rabbit non-immunized serum instead of the primary antibody.

2.4. Double immunofluorescence staining

Fresh frozen liver sections (10 μm in thickness) from control and CLD-treated rats on PI days 1 and 12 were used. Double immunofluorescence was carried out using CD163 in combination with CD68, Gal-3, MHC class II and CD204. Briefly, after fixation in cold acetone:methanol (1:1) for 10 min at 4 °C, the sections were incubated with 10% normal goat serum for 15 min. The sections reacted with the primary antibody overnight at 4 °C. After rinsing with PBS, the sections were incubated for 45 min with the secondary antibody goat anti-mouse IgG-conjugated with Alexa 488 or Alexa 568 (Invitrogen, Carlsbad, CA, USA) for CD68; and goat anti-rabbit IgG-conjugated with Alexa 568 (Invitrogen, Carlsbad, CA, USA) for Gal-3. The sections were then incubated with the primary antibody labeled with fluorescent dye conjugated secondary antibody: Alexa 488 labeled CD163 (AbD Serotec, Oxford, UK) for CD163/CD68; Alexa 488 labeled OX6 (AbD Serotec, Oxford, UK) for CD163/OX6; Alexa 488

Table 1
Primary antibodies used for immunohistochemistry.

Antibody	Type	Fixative	Dilution	Pretreatment	Source
CD68 (ED1)	Mouse monoclonal	PLP	1/500	MW in citrate buffer, 20 min	AbD Serotec, Oxford, UK
CD163 (ED2)	Mouse monoclonal	PLP	1/300	100 μg/ml Proteinase K, 10 min	AbD Serotec, Oxford, UK
CD204 (SRA-E5)	Mouse monoclonal	Zamboni's solution	1/1000	MW in citrate buffer, 20 min	Transgenic Inc., Kumamoto, Japan
MHC class II (OX6)	Mouse monoclonal	PLP	1/1000	MW in citrate buffer, 20 min	AbD Serotec, Oxford, UK
Iba-1	Rabbit polyclonal	Zamboni's solution	1/1000	MW in citrate buffer, 20 min	Wako Pure Chemical Industries, Osaka, Japan
Galectin-3 (Gal-3)	Rabbit polyclonal	Zamboni's solution	1/500	MW in citrate buffer, 20 min	Santa Cruz Biotechnology, Santa Cruz, CA, USA
BrdU	Mouse monoclonal	NBF	1/500	4N HCl, 30 min and 100 μg/ml Proteinase K, 10 min	Dako Corp, Glostrup, Denmark

PLP: periodate–lysine–paraformaldehyde; MW: microwave; BrdU: bromo-2-deoxyuridine; NBF: neutral buffered formalin