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Involvement of neutrophils in rat livers by low-dose thioacetamide administration

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ABSTRACT. The administration with high dose (close to LD_{50}) of thioacetamide (TAA), a hepatotoxicant used widely to induce experimental liver lesions, develops hepatocellular necrosis and subsequent inflammation (mainly M1-/M2-macrophages without neutrophil infiltration) in rats. We analyzed rat livers treated with a low dose TAA (50 mg/kg/body weight) at 6, 12, 18, 24 and 48 hr. The lesions in the affected centrilobular areas consisted of slight hepatocyte degeneration at 12 hr, and inflammatory cell infiltration at 18 and 24 hr; the lesions recovered until 48 hr. Translocation of intranuclei to cytoplasm of HMGB1, a representative molecule of damage-associated molecular patterns, was seen in some hepatocytes mainly at 6, 12, and 18 hr. As an interesting finding, at 12 hr, myeloperoxidase-positive neutrophil infiltration was observed in the affected centrilobular area. Additionally, CD68 M1-/CD163 M2-macrophages increased consistently at 12 to 48 hr. CXCL1, a chemokine for induction of neutrophils, began to increase at 6 hr and gradually increased at 12, 18 and 24 hr, apparently corresponding to the appearance of neutrophils. Collectively, the present findings at the low dose TAA indicated that along with M1-/M2-macrophages, neutrophils were characteristically seen, which might be elicited by cytoplasmic translocation of HMGB1 from nuclei. These finding would be useful for evaluation of hepatotoxicity at the early stages.

KEY WORDS: damage-associated molecular patterns (DAMPs), high-mobility group box 1 (HMGB1), myeloperoxidase, neutrophils, thioacetamide

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Neutrophils are one of the immune cells originated from and matured in the bone marrow [16]. Generally, when animals are infected with pathogens, particularly bacteria, neutrophils are recruited to the infected site as the first innate immune cells from bloodstream rapidly, in order to kill pathogens via various ways including phagocytosis, reactive oxygen species and neutrophil extracellular traps [16]. In sterile settings, the recruitment of neutrophils is well-studied in hepatic ischemic/reperfusion injury and they mediate the progression at the later stage after reperfusion injury [21]. Infiltration/migration of neutrophils in the liver may be intermediated by Kupffer cells via activating compliments and releasing chemokines including C-X-C motif chemokine ligand (CXCL)-1 and CXCL-2 [2, 14]. When cells are injured or undergo necrosis by ischemia or chemical exposure, damage-associated molecular patterns (DAMPs) are released to the extracellular space and can activate innate immune system through complicated mechanisms [8, 11, 13, 15]. As a representative DAMPs, high-mobility group box 1 (HMGB1), a non-histone binding protein participating in DNA transcription [1, 17], plays important roles in inflammation [7, 13].

Participation of neutrophils in chemically-induced liver injury is known in limited chemicals such as halothane [20] and acetaminophen [9, 10]. Thioacetamide (TAA) has been used to induce hepatotoxicity in rats and mice; the liver lesions induced by TAA are characterized by coagulation necrosis of hepatocytes in the centrilobular area followed by macrophage infiltration. The dose of TAA used for intraperitoneal injection on rats is usually 300 mg/kg body weight, of which dose is close to lethal dose 50 (LD_{50}) [5, 8]. In the present study, we analyzed pathological lesions induced in rat livers by a lower dose of TAA administration, focusing on neutrophils and HMGB1.

MATERIALS AND METHODS

Animals

Six-week-old, male F344/DuCrlCrlj rats were purchased from Charles River Japan (Yokohama, Japan). The TAA group was injected intraperitoneally with TAA dissolved in saline (50 mg/kg body weight; Wako Pure Chemicals, Osaka, Japan). The dose

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(50 mg/kg) was decided based on data in preliminary experiments. The control group was administered an equal volume of saline. These animals were housed in an animal room at a controlled temperature of 22 ± 3°C and with a 12-hr light-dark cycle; they were provided a standard diet (DC-8; CLEA, Tokyo, Japan) and tap water *ad libitum*. Rats were euthanized by deep isoflurane anesthesia, and the blood (from the abdominal artery) and liver were collected at 6, 12, 18, 24 and 48 hr after injection (n=4 in each point). Aspartate transaminase (AST) and alanine transaminase (ALT) were measured by SRL Inc. (Tokyo, Japan). The animal experiments were conducted under the institutional guidelines approved by the ethical committee of Osaka Prefecture University for animal care (No. 29-5).

Histopathology and immunohistochemistry

Tissues from the left lateral lobe of the liver were fixed in 10% neutral buffered formalin or periodate-lysine-paraformaldehyde (PLP) solutions. These tissues were dehydrated and embedded in paraffin. Deparaffinized sections, cut at 4 µm in thickness, were stained with hematoxylin and eosin (HE) for histopathologic examination. Immunohistochemical conditions were conducted according to methods reported previously [3]. PLP-fixed sections were used for immunohistochemistry with mouse monoclonal antibodies: cluster of differentiation (CD) 68 (clone ED1 for M1 macrophages; 1:500; Chemicon, Tokyo, Japan), CD163 (clone ED2 for M2 macrophages; 1:500; AbD serotec, Oxford, UK) and myeloperoxidase (for neutrophils; 1:500; R&D Systems, Minneapolis, MN, USA). After pretreated by microwave for 20 mins in 0.01 M citrate buffer (pH 6.0) for myeloperoxidase or by proteinase K (100 µg/ml) for 10 mins for CD68 and CD163, sections were incubated with each primary antibody for 1 hr at room temperature, followed by 1-hr incubation with peroxidase-conjugated secondary antibody (Histofine Simple Stain MAX-PO; Nichirei, Tokyo, Japan). Positive reactions were detected with 3, 3′-diaminobenzidine (DAB Substrate Kit; Nichirei). Sections were counterstained lightly with hematoxylin. In addition, liver samples obtained from rats injected with the high dose of TAA (300 mg/kg/body weight) were used for immunohistochemistry for myeloperoxidase.

Immunofluorescence

PLP-fixed sections were also used for immunofluorescence with rabbit polyclonal antibody for HMGB1 (1:500; Abcam, Cambridge, UK). After pretreated by microwave for 20 min in 0.01 M citrate buffer (pH 6.0), sections were incubated with the primary antibody for 24 hr at room temperature, followed by 1-hr incubation with fluorescence-conjugated secondary antibody (Alexa Fluor 488; Thermo Fisher Scientific, Waltham, MA, USA). Samples were mounted with medium including 4′ 6-diamindino-2-phenylindole (DAPI) for nuclear fluorescence.

Cell count

The numbers of myeloperoxidase, CD68 or CD163-positive cells in the affected centrilobular area was counted in different three areas of 4 different rats using WinROOF (Mitani Corp., Fukui, Japan) and are expressed as the number of positive cells per unit area (cells/mm^2).

Real-time PCR

Liver samples were immersed in RNAlater reagent (Qiagen GmbH, Hilden, Germany) overnight at 4°C and stored at −80°C. Total RNA was extracted by SV total RNA isolation system (Promega Corporation, Madison, WI, USA). Two µg of total RNA was reverse-transcribed with Superscript VILO reverse transcriptase (Life Technologies, Carlsbad, CA, USA). Real-time PCR was performed with TaqMan gene expression assays (Life Technologies) in PikoReal Real-Time 96 PCR System (Thermo Scientific). The TaqMan probes specific for the cytokines were used as follows (Assay IDs): cxcl1 (CXCL1), Rn00578225_m1; il6 (interleukin-6 (IL-6)), Rn01410330_m1; tnfa (tumor necrosis factor-α (TNF-α)), Rn99999017_m1; il1b (IL-1β), Rn00580432_m1; tgfb (transforming growth factor-β1 (TGFβ1)), Rn01440674_m1; ribosomal 18s (18s), Hs99999901_s1. The data were analyzed with the $2^{-\Delta\Delta C}{}_{T}$ method.

Statistics

Obtained data are represented as mean ± standard deviation (SD). Statistical analyses were performed using Dennett's test (versus control group). Significance was accepted at P<0.05.

RESULTS

The low dose TAA (50 mg/kg body weight) injection induces hepatocellular degeneration and inflammation

In livers of control (Fig. 1A) and at 6 hr, no histopathological abnormalities were seen. At 12 hr, some hepatocytes in the injured centrilobular area showed slight degeneration (Fig. 1B, arrowhead), with a small number of inflammatory cells. At 18 and 24 hr, inflammatory cell infiltration was more prominent in the affected area (Fig. 1C). At 48 hr, the lesions almost recovered. The inflammatory cells consisted of neutrophils and macrophages, specified by immunohistochemical analyses as mentioned below.

Being consistent with the histopathologic lesions, the AST and ALT values began to increase at 12 hr, with a peak at 24 hr. Both of the ALT and AST values showed a statistical increase at 18 and 24 hr, and the increased values were decreased at 48 hr (Fig. 1D for ALT; data not shown for AST).

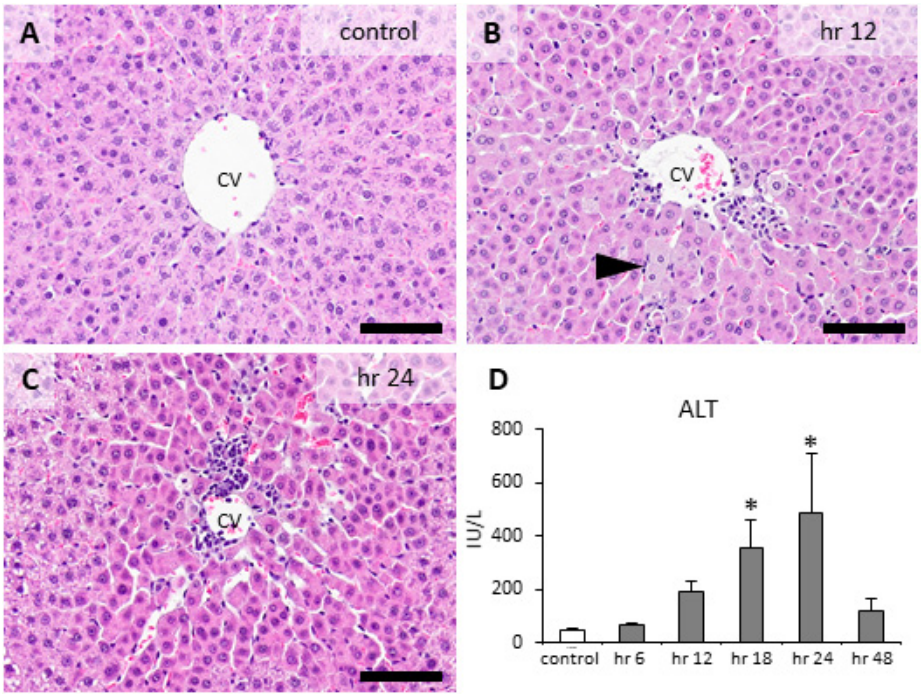

Fig. 1. Histopathology of control and thioacetamide (TAA) (50 mg/kg) treated livers. (A) Control liver shows normal hepatic architecture. (B) At 12 hr, degenerative change of some hepatocytes is observed in the affected centrilobular area with a small number of inflammatory cells (arrowhead: degenerative hepatocytes). (C) Inflammatory cells are notably seen in the centrilobular area at 24 hr. (D) Alanine transaminase (ALT) in TAA-treated rat livers after a single injection (50 mg/kg). The levels increase at 12, 18 and 24 hr, with a peak at 24 hr. A, B, C, hematoxylin and eosin. CV, central vein; bar=50 µm. *, $P<0.05$; Dunnett's test, vs. control.

Nuclear to cytoplasmic translocation of HMGB1 occurs in the early stage of low dose TAA-induced hepatic lesions

In the control liver, positive reactivity for HMGB1 was seen exclusively within the nucleus (Fig. 2A and 2B). At 6 and 12 hr, some hepatocytes in the centrilobular areas showed cytoplasmic positivity for HMGB1 (Fig. 2C and 2D). Hepatocytes with cytoplasmic positivity for HMGB1 were still observed a 24 hr; they were rarely seen at 48 hr as in the control liver.

Myeloperoxidase-positive cells appear as an inflammatory cell, in correlation with increased CXCL1

In livers of control and at 6 hr, there were no myeloperoxidase-positive cells (neutrophils) (Fig. 3A and 3E). At 12 hr, in the injured centrilobular area, a few cells reacting to myeloperoxidase were present (Fig. 3B and 3E), and the number increased gradually at 18 and 24 hr, showing a significant increase at 24 hr (Fig. 3C and 3E). At 48 hr, the positive cells were rarely seen (Fig. 3D and 3E).

The expression level of CXCL1, a neutrophil-activating/chemotaxis chemokine [8, 18], began to increase at 6 hr and gradually increased at 12, 18 and 24 hr, with a peak at 18 hr and showing a statistical increase (Fig. 3F).

Infiltrating macrophages represent M1-/M2-phenotypes

To evaluate immunophenotypes of infiltrating macrophages, immunohistochemical analysis was performed using CD68 (for M1 macrophage) and CD163 (for M2 macrophage). In livers of control and at 6 hr, CD68-positive M1 macrophages were rarely seen in the centrilobular area. At 12 and 18 hr, the positive macrophages aggregated in the centrilobular area (Fig. 4A), and the increased number retained at 24 and 48 hr (Fig. 4B).

In livers of control and at 6 hr, CD163-positive M2 macrophages were seen along the sinusoid, indicating Kupffer cells, without an increase in number. At 12 and 18 hr, besides positive cells along the sinusoids, CD163-positive macrophages appeared in the centrilobular area (Fig. 4C), and the increased number of the positive cells retained at 24 and 48 hr (Fig. 4D).

M1-/M2-macrophage-related cytokines increase significantly after TAA injection

mRNA expressions of M1-macrophage-related cytokines such as IL-6 (Fig. 5A), TNF-α (Fig. 5B) and IL-1β (Fig. 5C), which are also known as pro-inflammatory cytokines, significantly increased at 18 hr (for IL-6 and TNF-α) and 24 (for IL-1β). M2-macrophage-related cytokine, particularly TGF-β1 also known as fibrogenic cytokine [19, 20], significantly increased at 24 hr (Fig. 5D).

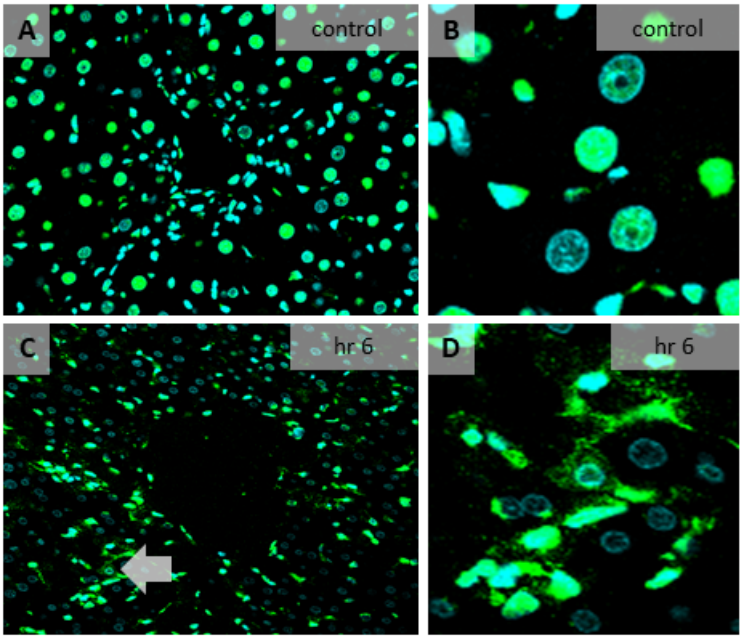

Fig. 2. Immunofluorescent images of control and thioacetamide (TAA) (50 mg/kg) treated livers for high-mobility group box 1 (HMGB1). (A) In the control liver, intrenuclear HMGB1 positivity is seen in hepatocytes at the basal level in the control. (B) An image at high magnification of (A). (C) At 6 hr, intracytoplasmic HMGB1 positivity is seen in some hepatocytes in the affected centrilobular area (arrow). (D) An image at high magnification of (C). Blue (DAPI); nuclei. Green; HMGB1.

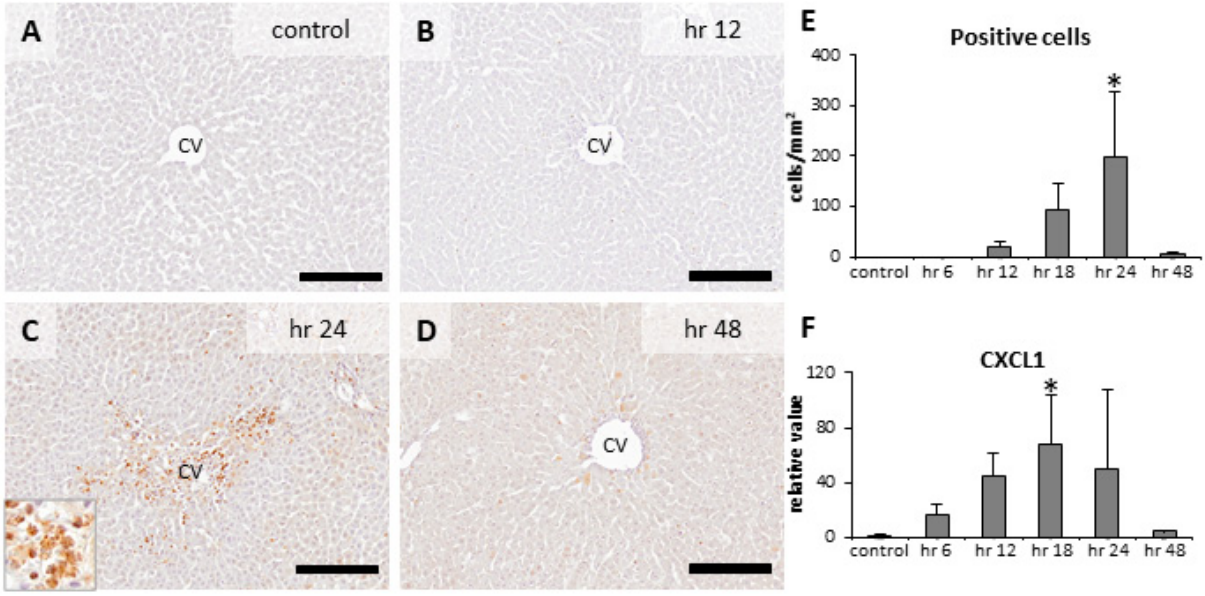

Fig. 3. Immunohistochemical analysis for myeloperoxidase as a marker of neutrophil in rat livers after TAA injection. (A) Myeloperoxidase-reacting neutrophils are not seen in control liver. (B) A few neutrophils are present in the centrilobular area at 12 hr. (C) Remarkable appearance of neutrophils is seen in the centrilobular area at 24 hr (inset: high magnification of positive cells). (D) A small number of neutrophils are seen in the affected areas at 48 hr. (E) The number of neutrophils tends to increase at 12, 18 and 24 hr, with a peak at 24 hr showing a statistical significance. (F) mRNA expression of neutrophil activating and chemoattracting chemokine, CXCL1, tends to increase at 12, 18 and 24 hr, showing a statistical significance at 18 hr. A, B, C, D, counterstained with hematoxylin. CXCL1, C-X-C motif chemokine ligand-1; CV, central vein; bar=50 µm. *, $P<0.05$; Dunnett's test, vs. control.

Myeloperoxidase-positive cells do not accumulate in the injured centrilobular area in rats administered with high dose TAA

To confirm relevance of neutrophil appearance with TAA administration, comparison on histopathological and immunohistochemical analysis was performed using high-dose TAA samples. In HE-stained sections, there were no

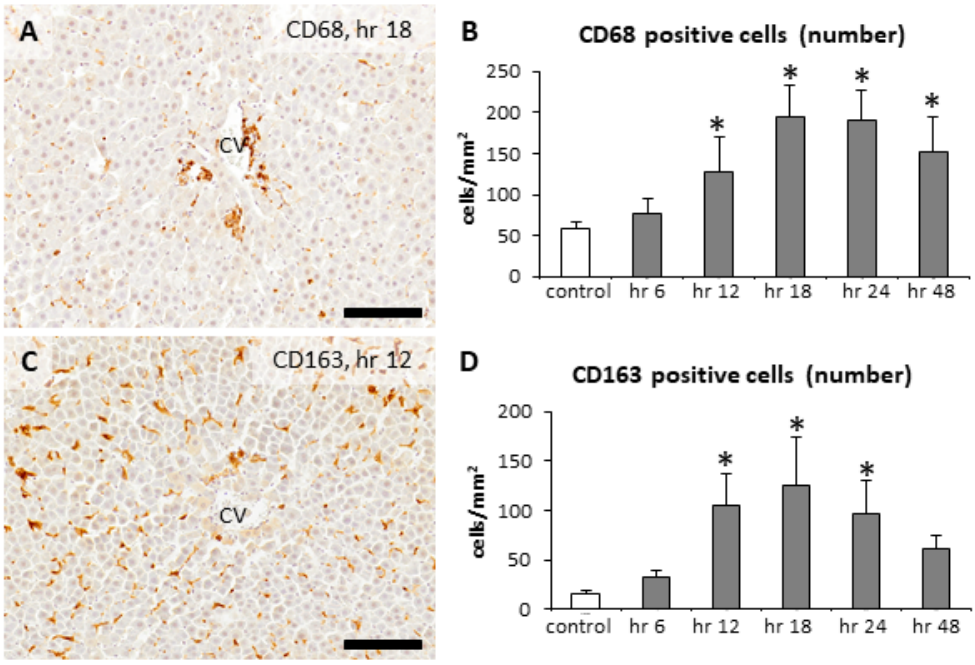

Fig. 4. Immunohistochemical analysis for CD68 for M1-macrophages and CD163 for M2-macrophages in rat livers after TAA injection. (A) CD68-positive macrophages are seen in the centrilobular area at 18 hr as aggregations. (B) The number of CD68-positive macrophage significantly increases at 12 hr onwards, with a peak at 18 hr. (C) There are many CD163-positive macrophages with swollen cytoplasm in the centrilobular area at 12 hr. (D) The number of CD163-positive macrophages significantly increases at 12 hr onwards, with a peak at 18 hr. A, C, counterstained with hematoxylin. CD, cluster of differentiation; CV, central vein; bar=50 µm. *, $P<0.05$; Dunnett's test, vs. control.

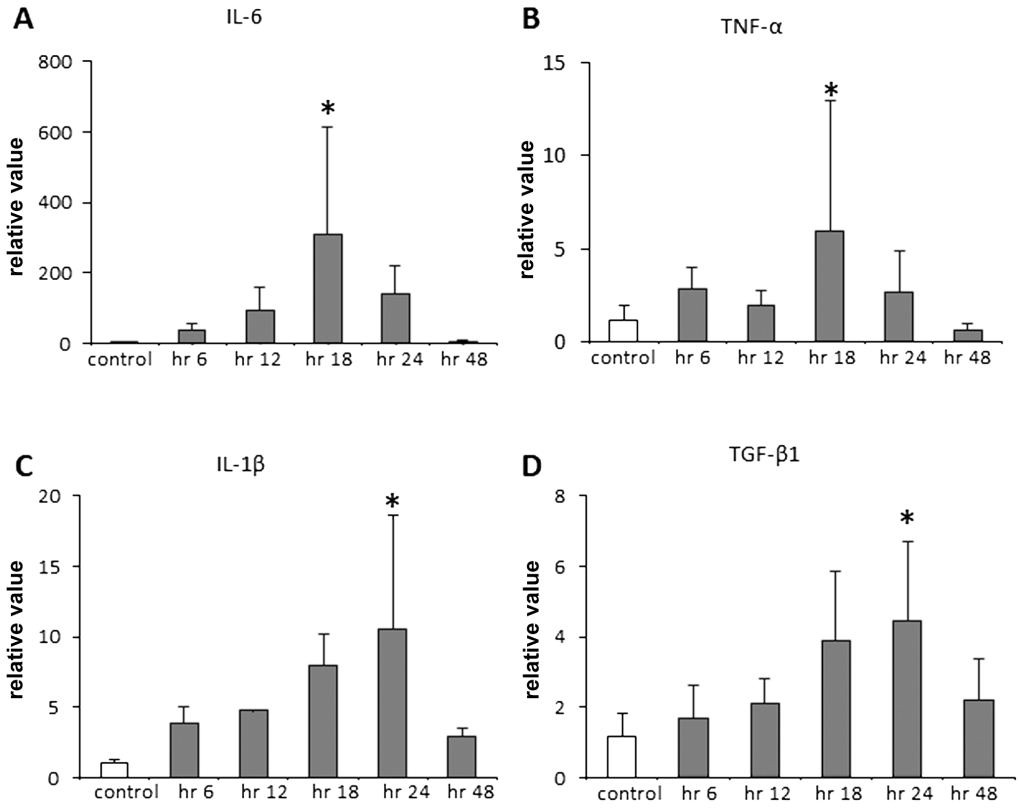

Fig. 5. mRNA expressions of M1-macrophage-related inflammatory cytokines such as (A) interleukin-6 (IL-6), (B) tumor necrosis factor-α (TNF-α) and (C) IL-1β, and M2-macrophage-related anti-inflammatory cytokine such as transforming growth factor-β1 (TGF-β1) (D). *, $P<0.05$; Dunnet's test, vs. control.

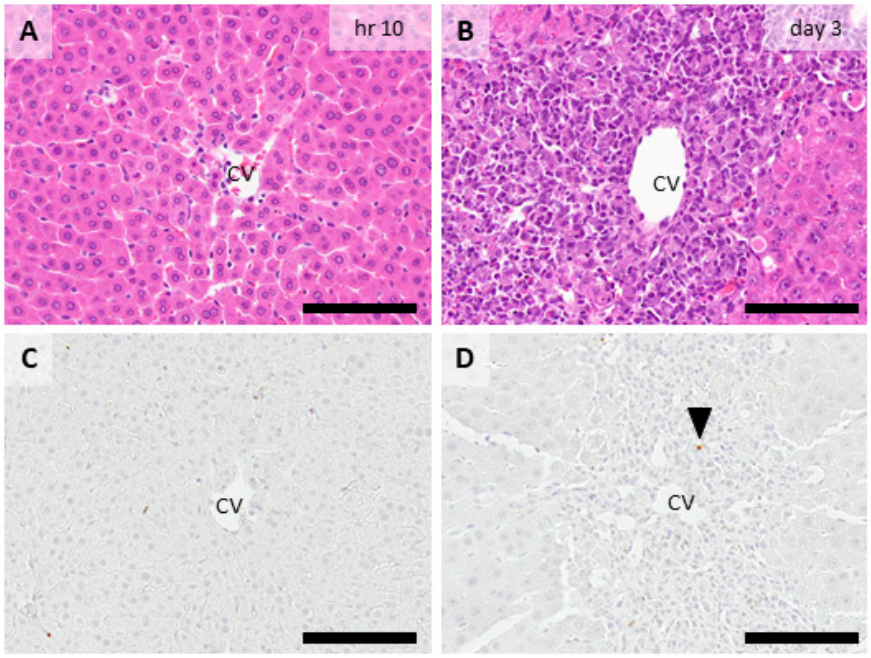

Fig. 6. (A and B) Histopathology of rat livers treated with the high dose (300 mg/kg/body weight) and (C and D) immunohistochemical analysis for myeloperoxidase as a marker of neutrophil. Previously reported samples were used [8]. (A) At 10 hr, hepatocyte injury is rarely seen and (C) there are no neutrophils reacting to myeloperoxidase. (B) On day 3, there are many inflammatory cells in the affected centrolobular area. (D) Nevertheless, neutrophils reacting to myeloperoxidase are rarely seen; the infiltrating cells are M1/M2 macrophages as previously reported [18]. A, B, hematoxylin and eosin; C, D, counterstained with hematoxylin. CV, central vein; bar=100 µm.

histopathological lesions in the centrilobular area at 10 hr (Fig. 6A), whereas many infiltrating cells were seen on day 3 (Fig, 6B). Not only at 10 hr (Fig. 6C) but also on day 3 (Fig. 6D), myeloperoxidase-positive neutrophils were rarely seen. The infiltrating cells were almost macrophages reacting to CD68 and CD163 as confirmed previously [18].

DISCUSSION

Injection of TAA at the present low dose (50 mg/kg body weight) induced hepatocyte degeneration and inflammatory cell reaction in the centrilobular area, with simultaneously increased values of AST and ALT. The procedure of histopathological changes were in accordance with those in rats injected at the TAA high dose (300 mg/kg body weight) [8], although the hepatic lesions were much milder in the present study than those in our previous studies with the high-dose TAA. The time course (injury/inflammation and recovery) was shorter than that in the high-dose study; the present low-dose injection developed lesions at 12 and 24 hr, and then the lesions almost recovered, whereas the high-dose administration induced hepatic lesions consisting of necrosis/inflammation on days 2 and 3, and subsequent recovery on day 5 [6, 8].

Interestingly, in the present low dose TAA administration, neutrophil infiltration was characteristically seen. The finding has not been reported in hepatotoxicity of the TAA high dose experiments [6, 8, 18]; to confirm the finding, we conducted immunohistochemistry for myeloperoxidase using liver samples in rats injected with the TAA high dose. Cells reacting to myeloperoxidase were rarely seen even on day 3 (Fig. 6D) when infiltration was the greatest. On the contrary, CD68-expressing M1 macrophages and CD163-expressing M2 macrophages appeared simultaneously in the affected liver lesions, as seen at the high dose TAA [4]. Of note, myeloperoxidase-positive neutrophils tended to increase mainly at 18 and 24 hr, whereas CD68 M1-/CD163 M2-macrophages increased consistently at 12 to 48 hr. These findings indicated that the appearance of neutrophils was transient in rat livers treated with at the low dose TAA.

CXCL1 is a chemokine for induction of neutrophils [2, 14]. Interestingly, CXCL1 mRNA began to increase at 6 hr and thereafter gradually increased at 12, 18 and 24 hr, apparently corresponding to the appearance of neutrophils, particularly at 12, 18 and 24 hr. CXCL1 may be produced by activated Kupffer cells. Increased number of CD163-expressing M2 macrophages (maybe including Kupffer cells reacting to CD163), which began to be seen at 12 hr (Fig, 4D), might have been related to the appearance of neutrophils.

In the present study, the translocation of intranuclear HMGB1 to cytoplasm was observed in some hepatocytes mainly at 6 and 12 hr. Possibly, neutrophil infiltration might be elicited by HMGB1. Releasing of HMGB1 to extracellular situation prompts to transcript pro-inflammatory factors, including IL-1β, IL-6 and TNF-α, through complicated innate immune systems [12, 17]. It would be worth to investigate the roles of HMGB1 to find out the mechanisms of neutrophil infiltration at the TAA low dose.

In conclusion, the present study showed that injection of TAA at the low dose (50 mg/kg body weight) could induce liver lesions following neutrophil infiltration, along with M1-/M2-type macrophages. The neutrophil infiltration might be related to the translocation of intranuclear HMGB1 to cytoplasm. Although the significance of neutrophils should be investigated at different time points using various hepatotoxicants including TAA, the analyses of HMGB1 translocation, and neutrophil appearance and its related factors (such as CXCL1) would be useful for evaluation of hepatotoxicity at the early stages.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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