Effect of Aging on Auto-Antibodies to Wounded Tissues

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Abstract: Wound healing succeeds tissue destruction. We hypothesized that antibodies might bind to wounded tissues, which would facilitate the engulfment of damaged tissues by macrophages. We detected the autoantibodies in sera of different ages of mice, which bind to wounded tissues. We detected IgG1 binding to wounded tissues by using FITC-labeled anti-IgG1 in C57BL/6 mice. These bands were highest in 4 months old C57BL/6 mice. The sera taken from 2 months and 20 months old mice also bound to wounded tissues, although the bands were weaker than those of 4 months old mice. We also examined the autoreactive IgM binding to wounded tissues. We could detect relatively strong bands even in 2 M old mice. The pattern of these bands was changed by advancing age. Intensity of IgM bands was not decreased by advancing age. The splenectomy reduced the intensity of IgG1 bands especially in 4 months old mice. Although we observed the slight delay of wound repair by splenectomy in 2 M and 20 M old mice, we observed that wound repair was strongly delayed in 4 M old mice. Serum from any age of mice enhanced the macrophage phagocytosis by opsonization. Serum taken from splenectomized mice decreased the opsonizing capacity only at 4 M.

Keywords: Aging, IgG1, wound healing, splenectomy.

INTRODUCTION

One of the most important clinical problems in caring for elderly patients is treatment of pressure ulcers. Pressure ulcers are caused by compression, friction, infection and/or malnutrition. The ability to heal pressure ulcers declines with age [1]. To understand the mechanism underlying the impaired ability of older patients to repair pressure ulcers, we established studies to analyze wound healing in aged mice. The wound repair process is a highly ordered sequence of events that encompasses hemostasis, inflammatory cell infiltration, and tissue re-growth and remodeling [2]. Initially, neutrophils accumulate at sites of injury, followed by a large influx of macrophages. The function of granulocytes and macrophages in wound healing and inflammation has been extensively studied [3-5]. Neutrophils have been shown to prevent wound repair processes in neutrophil depletion studies [6]. These studies were done using young mice. However, we have shown in aged mice that the depletion of neutrophils by anti-Gr-1 antibody dramatically delayed wound healing [7].

The main function of macrophages and neutrophils is phagocytosis of pathogens and cellular debris. These cells phagocytize apoptotic and necrotic cells directly through use of their phagocytic receptors [8]. Clearance of damaged tissue and debris proceeds more efficiently when autoantibodies attach to the wounded tissues (opsonization). There are several reports which describe the effects of T or B cells in wound healing, with the exception of γδ T cells [9, 10]. In aseptic wound healing, very few T or B cells migrate to the wound site. However, immunoglobulins secreted by B cells can reach the wound sites. Recently, in order to examine the effects of immunoglobulin, we performed splenectomy in a mouse model and found that it greatly delayed wound healing by reducing the amount of IgG1 binding to the damaged tissues [11]. To better understand the role of autoantibodies in the repair of pressure ulcers in older patients, we examined IgM and IgG1 autoantibodies bound to wounded tissues from both young and aged mice. Further, we examined whether wound healing was accelerated by these autoantibodies.

MATERIALS AND METHODS

Mice and Splenectomy

Two months (2M) old C57BL/6 female mice were purchased from SLC Japan and maintained throughout their lifespan in the Animal Research Facility at the Nagoya University Graduate School of Medicine under specific pathogen-free conditions and used according to institutional guidelines. Mice (4M, 6M, 12M, 17M, 20M, 26M old) were also maintained there. The aged mice were free of cancer and other diseases. In splenectomy experiment, mice of different ages (2M, 4M, 20M) were anesthetized and

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1874-8279/10

subjected to either a sham operation or splenectomy. One week later, punch biopsies were performed on the backs of these mice.

Punch Biopsy Wounding and Macroscopic Examination

After shaving and extensive cleaning with 70% ethanol, the dorsal skin was picked up at the midline and two layers of skin were punched through with a sterile disposable biopsy punch (diameter 3 mm; Kai Industries). This procedure generated two excision full-thickness wounds with one on each side of the midline. The same procedure was repeated four times, generating eight wounds on each animal. Each wound site was digitally photographed at the indicated time intervals, and wound areas were determined on photographs using Photoshop (version 7.0; Adobe Systems) and the areas were calculated with Excel. Changes in the areas of wounded sites were expressed as the proportion of the initial wound areas. For Western blotting, wounds and their surrounding areas (including the scab and epithelial margins) were cut for further analyses with a sterile disposable biopsy punch with a diameter of 6 mm (Kai Industries) at the indicated time points.

Auto-Antibody Detection by Western Blotting

Tissues were homogenized in buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 2 mM EDTA-2Na), and then lysed in sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 2.5% glycerol, 5% 2-mercaptoethanol, and 0.05% bromophenol blue). Total protein (1 mg/mL) was resolved by SDS-PAGE and then transferred to PVDF membranes (Millipore). Blotted membranes were reacted with 1/100 diluted serum. Then, membranes were reacted with goat anti-mouse IgG1 secondary antibodies or anti-mouse IgM secondary antibodies (Santa Cruz). Bound antibodies were detected with an enhanced chemiluminescence (ECL) system (Amersham Pharmacia). The intensity of bands in Western blots was determined by image analysis software (Lumi Vision Analyzer 400 product by AISHIN).

Phagocytosis by Macrophages

Three days after injection of 1 mL of 3% thioglycollate medium, peritoneal macrophages were collected from groups of three mice and were isolated by adherence of the cells to tissue culture plates overnight at 37°C in 5% CO_2. Murine peritoneal neutrophils were collected as described before by injecting 0.5 mg zymosan intraperitoneally [7]. Neutrophils were treated with CFSE, then damaged by 10 sec exposure to 100°C heat. Macrophages (5×10^5) and targets (damaged neutrophils) were plated at a ratio of one macrophage to one target cell. The mixture was incubated at 37°C in 5% CO_2 for 1 h and washed with PBS to remove unincorporated target cells. They were detached by trypsin/EDTA treatment. Cells were assessed on a flow cytometer (FACSCalibur, Becton Dickinson), and analyzed using FlowJo software (Tomy Digital Biology).

RESULTS

IgG1 and IgM Binding to Wounded Tissues: Age Differences

Although we detected distinct bands in 2M old mice, the intensities and varieties of bands from 2M old mice were low. The intensity of the bands was greatly increased at 4M. Although the pattern of these bands changed with advancing age, we could detect specific bands during aging. In previous paper, we specified some of the bands by mass spectroscopy [11]. Because each bands in this paper may contain another proteins, we tentatively named the bands by molecular weight. We measured the intensity of each band observed in the gels. Many bands gradually increased with advancing age, peaking around 17M old (220K, 150K and 100K) or 12M old (43K and 75K). Almost all bands decreased at 20M of age (Fig. **1**). When we examined auto-reactive IgM binding to damaged tissues, we detected relatively strong bands, even in 2M old mice. The pattern of these bands changed during aging. The intensity of IgM bands did not decrease over time (Fig. **2**).

Age-Dependent Reduction in the Binding of IgG1 Antibodies to Damaged Tissues after Splenectomy

Because the IgG1 antibody binding pattern to wounded tissues changed with advancing age, we examined the effects of splenectomy. At 4M of age, the IgG1 bound to wounded tissues was dramatically decreased by splenectomy. However, the IgG1 bound to damaged tissues in 20M old C57BL/6 mice decreased less than that observed with 4M old mice following splenectomy (Fig. **3A**). By gel intensity analysis, we found that almost all bands (220K, 150K, 100K, 75K and 43K) were greatly decreased by splenectomy at 4M. These bands were decreased in 20M old mice. However, in splenectomized 2M old mice, we observed only a slight decrease of 220K, 150K, 100K, 75K (Fig. **3B**).

Delayed Wound Repair in Splenectomized Mice is Age-Dependent

Two-month, 4M or 20M old C57BL/6 mice were splenectomized or given sham operations. At one week post-operation, we performed punch biopsies on the backs of these mice. Although we observed a slight delay of wound repair following splenectomy in 2M and 20M old mice, we observed that wound repair was strongly delayed in 4M old mice (Fig. **4**).

Effects of Aging on Opsonization

Antibodies to the damaged tissues might facilitate phagocytosis by opsonization of the damaged tissues. Therefore, we asked whether serum antibodies might enhance phagocytosis by macrophages. We used neutrophils as damaged cells in this experiment. We found that the addition of serum to heat-damaged neutrophils facilitated phagocytosis by macrophages. The extent of phagocytosis without serum was 13.3%. The percentages of phagocytosis with serum from 2M old, 4M old and 20M old mice were 36.0%, 45.2% and 39.7%, respectively. These results indicate that opsonization by serum greatly enhances wound healing at all ages. Only serum taken from splenectomized mice at 4M decreased the opsonizimg capacity. The opsonizing capacity of the serum taken from splenectomized mice at 2M or 20M old did not significantly differ from that of sham-operated mice (Fig. **5**).

DISCUSSION

Here we have shown that the pattern of IgG1 antibodies targeted against damaged tissues changed with advancing

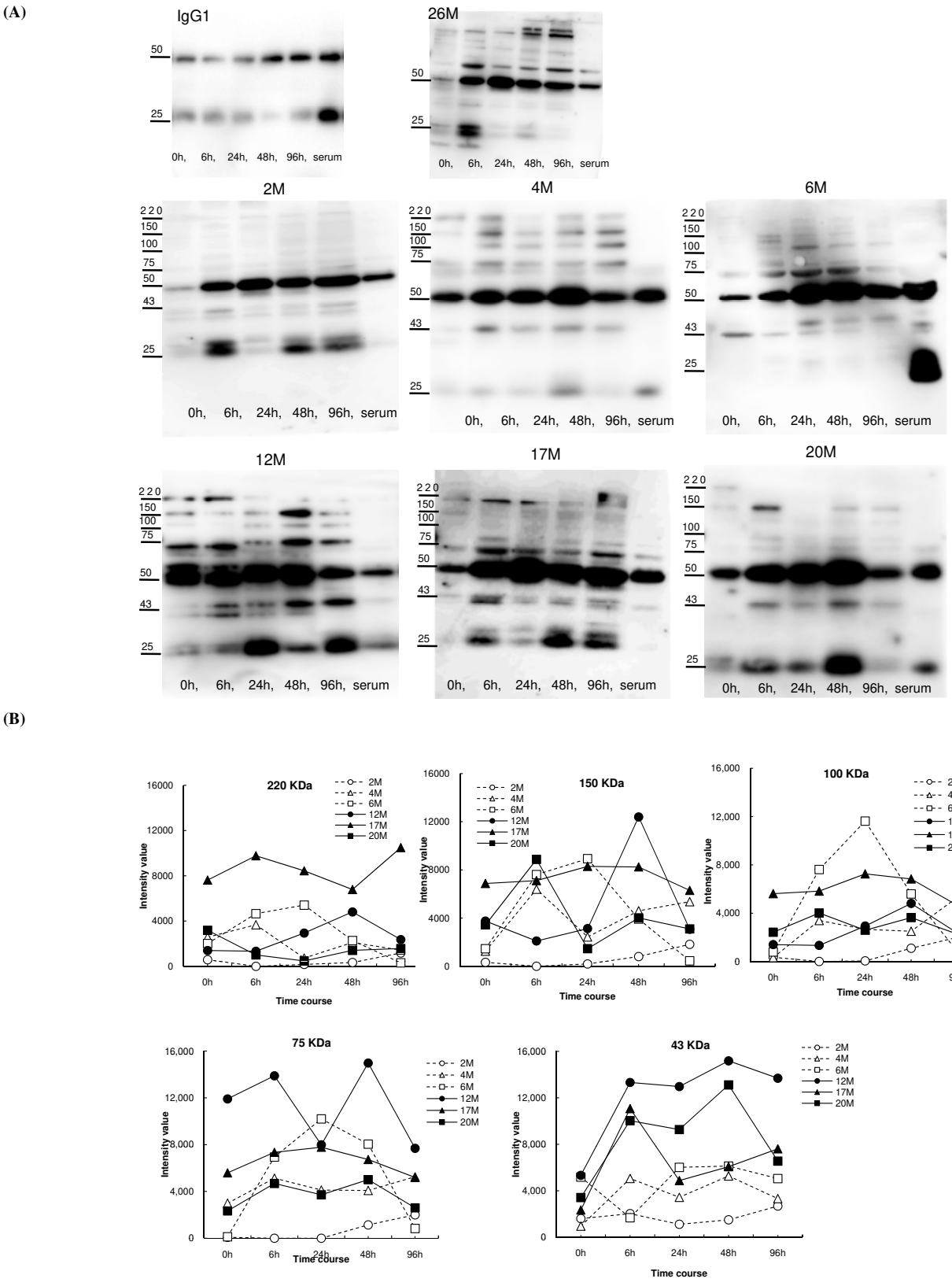


Fig. (1). Age-related changes of IgG1 auto-antibodies bound to damaged skin.
Damaged tissues were taken from 2M old C57BL/6 mice at 0 h, and 6 h to 96 h after punch biopsy. Tissue lysates/homogenates were subjected to SDS PAGE and transferred to PVDF membranes. Sera (diluted 1:100) taken from 2M, 4M, 6M, 12M, 17M, 20M or 26 M old mice were reacted with membranes as a source of primary antibodies and then the membranes were reacted with anti-mouse IgG1 secondary antibodies. **A.** Bound antibodies were detected by the enhanced chemiluminescence (ECL) system. Representative results are shown for the sera taken from five mice at each age. Lines indicate the possible bands for quantitative analysis. **B.** Determination of band intensity by image analysis software. Intensity of each band was determined after subtracting background intensity around the band.

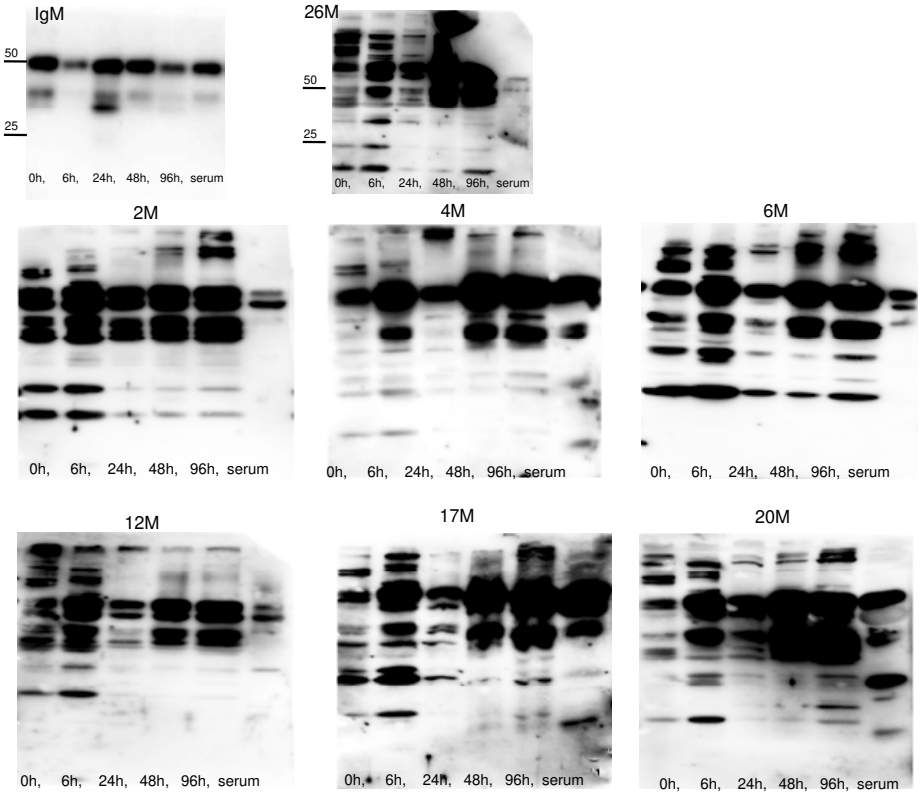
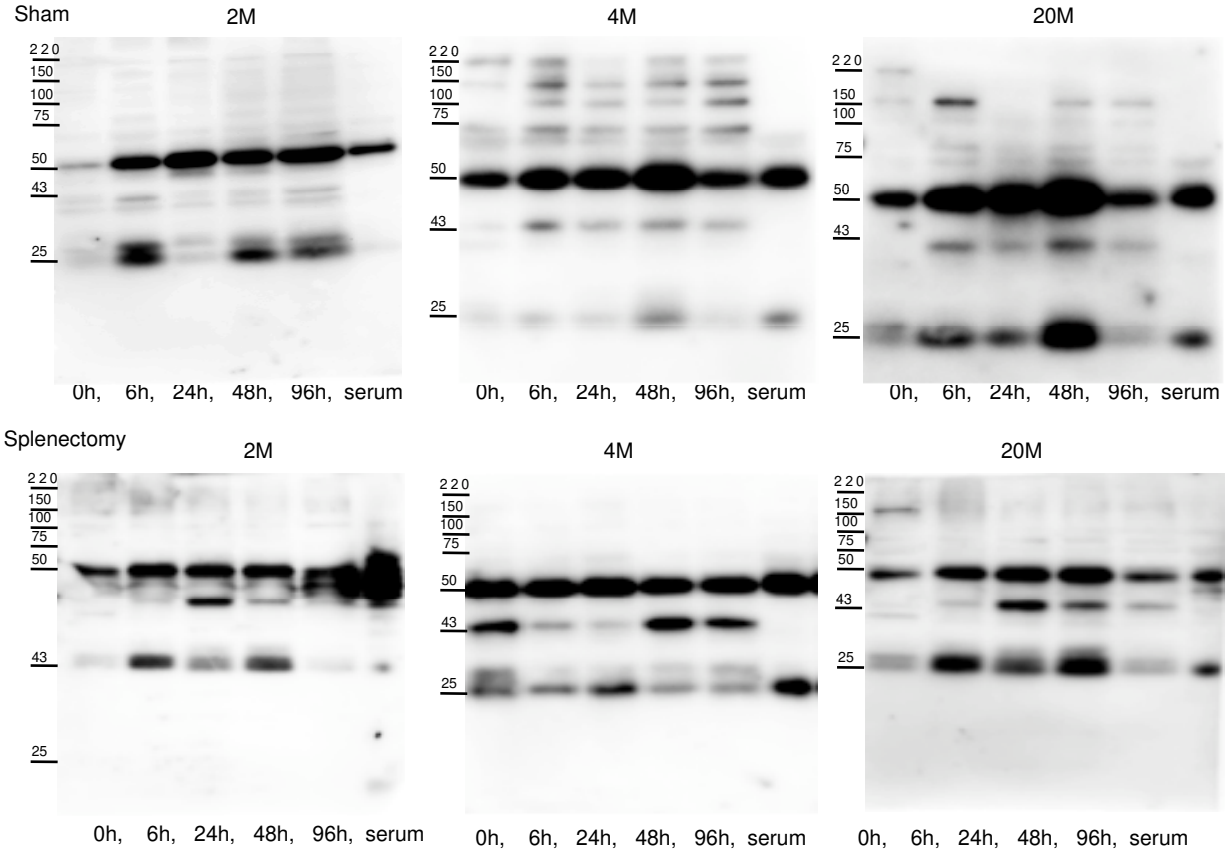


Fig. (2). Age-related changes of IgM auto-antibodies bound to damaged skin.
Damaged tissues were taken from 2M old C57BL/6 mice at 0 h, and 6 h to 96 h after punch biopsy. Tissue lysates/homogenates were subjected to SDS PAGE and transferred to PVDF membrane. Sera (diluted 1:100) taken from 2M, 4M, 6M, 12M, 17M, 20M or 26 M old mice were reacted with membranes as a primary antibody and then membranes were reacted with anti-mouse IgM secondary antibodies. Bound antibodies were detected by the enhanced chemiluminescence (ECL) system. Representative results are shown for sera taken from five mice at each age.

(A)



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(B)

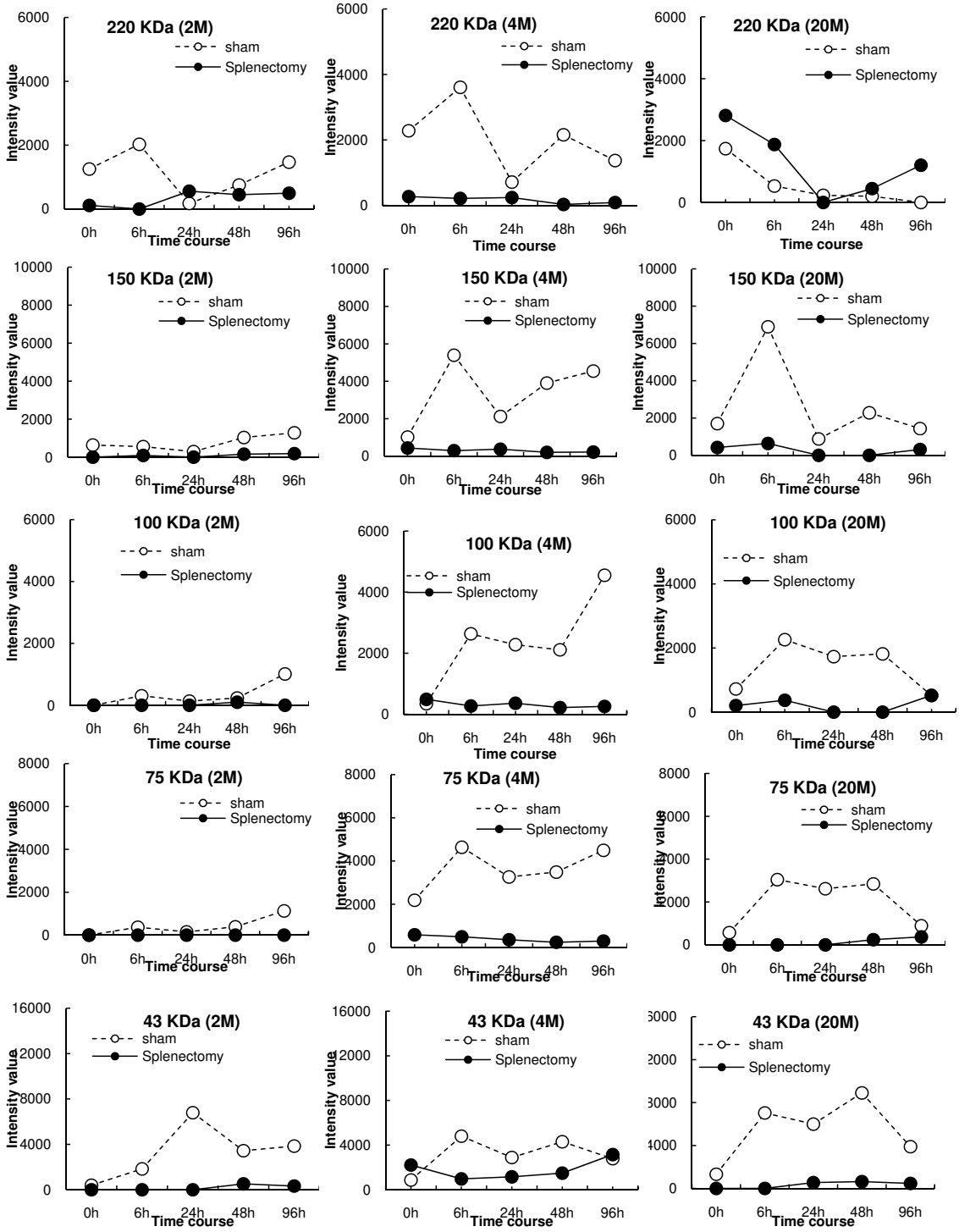


Fig. (3). Age-dependent reduction of IgG1 antibody binding to damaged tissues following splenectomy.
Damaged tissues were taken from 2M old C57BL/6 mice at 6 - 96 h after punch biopsy. Tissue lysates/homogenates were subjected to SDS PAGE and transferred to PVDF membranes. Sera were collected from 2M, 4M or 20M old mice one week after a sham operation or splenectomy. Sera (diluted 1:100) were reacted with membranes as a source of primary antibodies and then membranes were reacted with anti-mouse IgG1 secondary antibodies. **A.** Bound antibodies were detected by the enhanced chemiluminescence (ECL) system. Representative results are shown for sera taken from five mice at each age. Lines indicate possible bands for quantitative analysis. **B.** Determination of band intensities by image analysis software. Intensity of each band was determined after subtracting background intensity around the band.

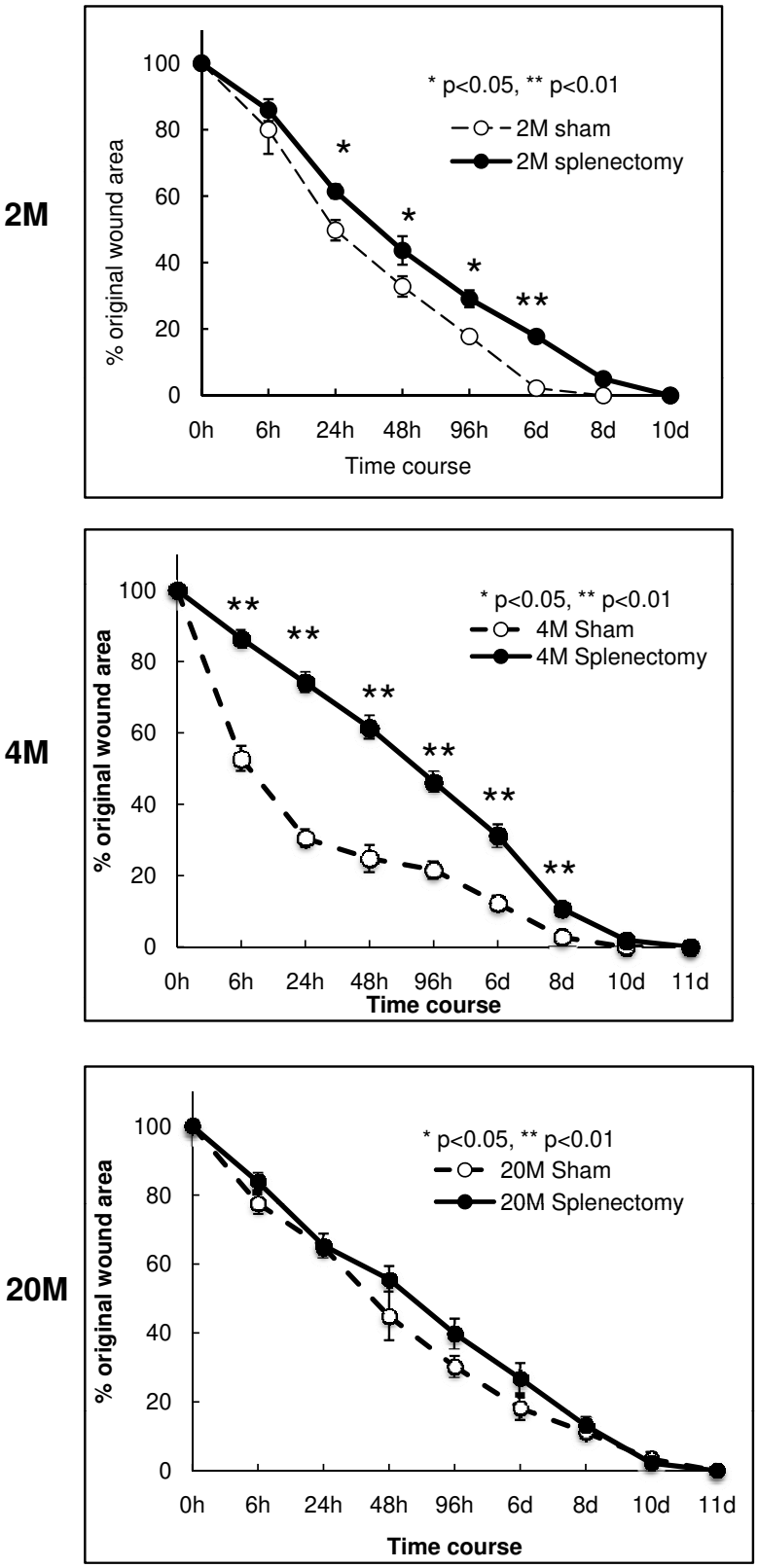
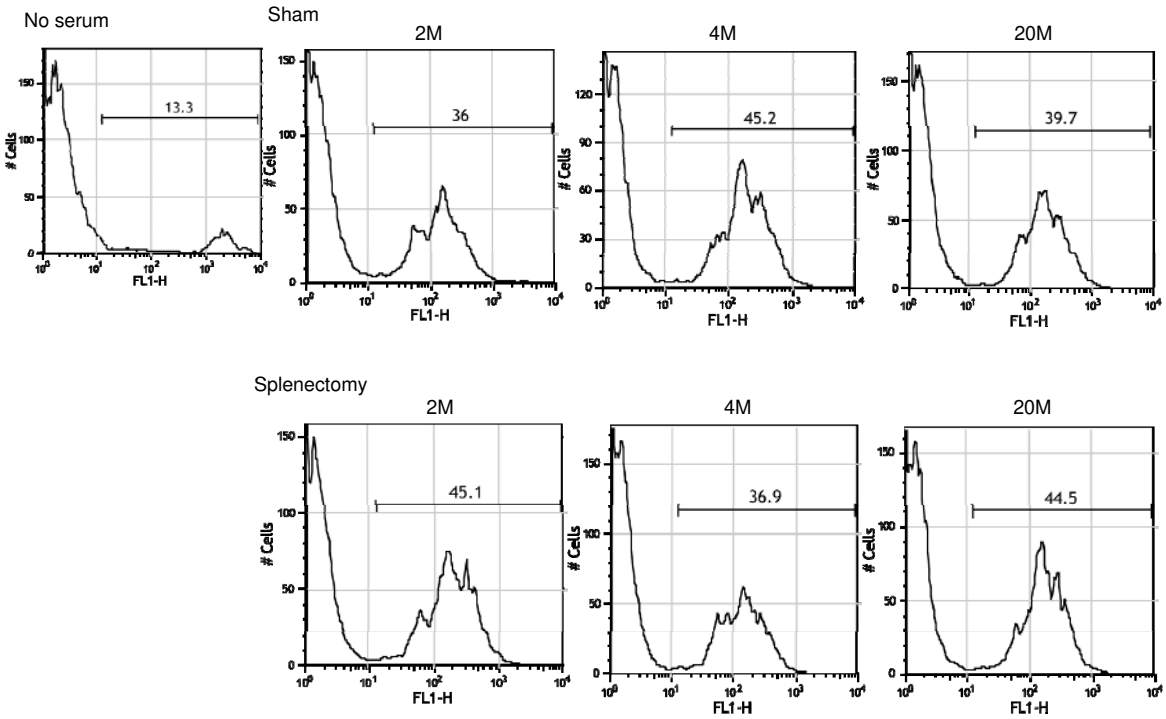


Fig. (4). Delayed wound healing in splenectomized C57BL/6 mice.
Two month, 4M or 20 M old C57BL/6 mice were splenectomized or subjected to a sham operation. One week later, punch biopsies were performed on the backs of these mice. Changes in the percentages of wound areas are shown at each time point in comparison to the original wound area in splenectomized (closed circles) or sham-operated mice (open circles). Data shown are the mean ratios ± SEM of five mice. (*; $p < 0.05$, **; $p < 0.01$).

(A)



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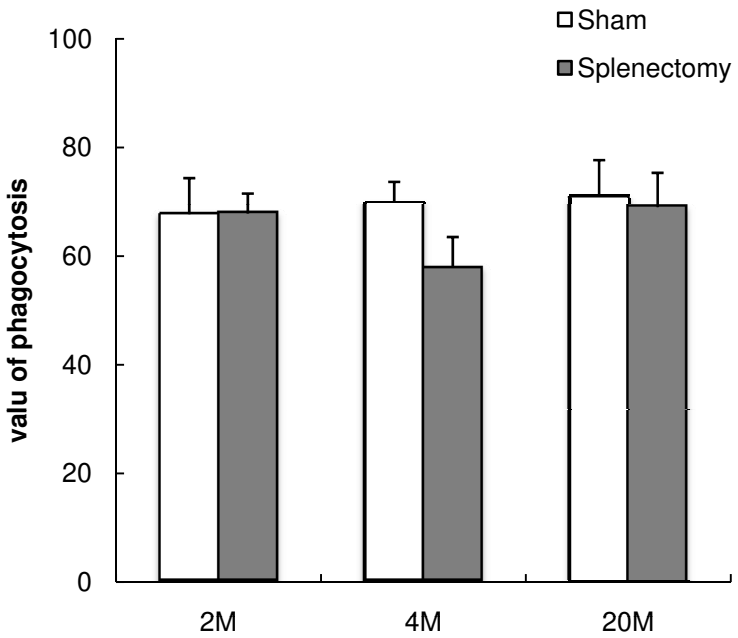

Fig. (5). Serum from aged mice had the same level of opsonizing capacity as that of young mice.
Neutrophils were taken from C57BL/6 as described in Materials and Methods. Serum samples from 2M, 4M or 20M old C57BL/6 mice (sham operated; upper lane, splenectomized; lower lane) were added to heat-treated neutrophils and CFSE-labeled. They were phagocytosed by macrophages taken from C57BL/6 as described in Materials and Methods. **A.** Representative results of FACS experiments. **B.** Effects of opsonization. Data were calculated as follows. Value= (% phagocytosis with serum- % phagocytosis without serum) / (% phagocytosis with serum). Data were derived from five experiments using sera taken from five mice at each age.

age. The number and intensity of IgG1 bands bound to wounded tissues at 2M were lower than those of 4M old C57BL/6 mice. Although the number and intensity of IgG1 bands bound to wounded tissues increased until 12M or 17M of age, those from 20 M old C57BL/6 mice decreased (Fig. **1**). Splenectomy strongly reduced IgG1 binding to wounded tissues at 4M of age. Although the IgG1 bands were reduced by splenectomy at 2 M and 20M of age, the reduction was less than that observed in 4M old mice (Fig. **3**). These findings correlated with the prominent delay of wound healing

by splenectomy at 4M of age. Shorter delays of wound healing following splenectomy were seen in mice at 2M and 20M of age (Fig. **4**).

We have shown that both IgG1 and IgM antibodies bind to damaged tissues. IgM antibodies to wounded tissues appeared early in life and remained throughout the life of the mice (2 M to 20 M) (Fig. **3**). IgM, IgG and IgA autoantibodies, reactive with a variety of serum proteins, cell surface structures and intracellular structures, are "naturally" found in all normal individuals (natural autoantibodies, NAAs) [12]. The exact cellular origin of NAAs is unclear. Mature B cells can be divided into two major populations: B1 and B2 cells [13]. B2 cells in the spleen are further divided into two subsets, follicular (FO) and marginal zone (MZ) B cells [14]. Because B1 cell-derived Abs bear similarity to NAAs in that they often recognize self-Ags, it is believed that B1 cells are a major source of NAAs [13, 15, 16]. However, several reports have shown recently the existence of IgG antibodies to auto-antigens. It has been shown that B cells with a low level of self-reactivity or with reactivity to bacterial wall components preferably reside in the MZ [17]. Although B cells are subject to negative selection, B cells can be positively selected, generated and maintained on the basis of their auto-reactivity [18]. Thus, autoreactive, autoantibody-secreting B lymphocytes are found in healthy individuals and are stimulated by auto-antigens. NAAs constitute a large fraction of serum immunoglobulin. Immunoadsorption experiments showed that up to 66% of IgG from individual sera were auto-reactive [19, 20]. Because class switching from IgM to IgG occurs in secondary lymph organs such as the spleen, it is reasonable that splenectomy reduces the production of autoreactive IgG antibody.

Our hypothesis is that IgG1 opsonizes the damaged tissues for subsequent phagocytosis. In order to examine phagocytosis, we used neutrophils as target tissue cells, which were phagocytosed by macrophages. In these experiments, we obtained neutrophils and macrophages from 4M old mice. Because opsonizing capacity of antibodies is mainly caused by IgG1, the peak reduction of IgG1 by splenectomy at 4M may be correlated to the decrease of opsonising capacity (Fig. **5**). The reason for similar levels of opsonization from 2M and 20M old mouse sera compared to 4M old mouse serum cannot be explained only by IgG1 binding. Complement, which is activated by IgM or IgG antibodies (other than IgG1) may also induce opsonization.

Taken together, we have show in this paper that IgG1 opsonizes the damaged tissues, which are removed by phagocytosis by macrophages. This IgG1 dependent opsonization is highest at 4M old mice, because splenectomy reduces phagocytosis and wound healing only at 4M old mice.

ACKNOWLEDGEMENT

We thank Mr. Tanaka in the Division for Medical Research Engineering, Nagoya University Graduate School of Medicine for maintenance of the flow cytometer. This work was supported by the Ministry of Education, Science, Technology, Sports and Culture, Japan.

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Received: March 17, 2010 Revised: April 27, 2010 Accepted: April 30, 2010