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Immunohistochemical study on a macrophage calcium-type lectin in mouse embryos: transient expression in chondroblasts during endochondral ossification

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We investigated expression of mouse macrophage galactose/*N*-acetylgalactosamine-specific calcium-type lectin (MMGL) in mouse embryos using a rat monoclonal antibody (mAb) LOM-14 that we previously developed. Immunoblot analysis revealed that a significant expression of MMGL was first detected in detergent extracts of whole embryos of 11 days post coitus (dpc) and the level of its expression increased during further fetal development (examined up to 18-dpc embryos). Tissue sections of 12, 14, 16, and 18-dpc embryos, newborn and adult mice were investigated by immunohistochemical staining. In embryos of 12-dpc and later stages, mesenchymal cells (typically distributed in the embryonic skin) exhibited positive signals for MMGL. Interestingly, a conspicuous staining was observed during endochondral ossification in temporary cartilage tissue, in which chondroblasts were transiently positive for MMGL. The staining intensity for the chondroblasts peaked in 14-dpc embryos and then gradually decreased. The staining was diminished while hypertrophy and maturation of chondrocytes proceeded, and was eliminated in areas with calcification. Immunoelectron microscopic study demonstrated the presence of MMGL in rough endoplasmic reticulum in the chondroblasts in the temporary cartilage tissue in 14-dpc embryos. These results provide first evidence showing the expression of MMGL in cells other than macrophages.

***Keywords*: calcium-type lectin, macrophage marker, embryo, chondroblasts, endochondral ossification, immunohistochemistry, mouse**

Introduction

Macrophages are among the earliest hemopoietic cells to appear in embryonic development. They are thought to play important roles in organogenesis, tissue remodeling and hemopoiesis, however, their detailed functions are not fully understood. Recently, monoclonal antibodies (mAbs) specific for macrophage population have been developed and have served to provide information on macrophages during fetal development. F4/80 antigen, defined by one of such mAbs [1], has been described as a mature macrophage marker found in monocytes as well as in a wide variety of tissue macrophages [2]. During mouse development, it has been reported that F4/80-positive cells were first detected in yolk sac, fetal liver and mesenchyme in 10-days post coitus (dpc) embryos, and subsequently there was expansion of this population in liver, spleen and bone marrow in association with the initiation of hemopoiesis in these organs in 11, 15 and 17-dpc embroys, respectively [3]. Since F4/80-positive stromal macrophages detected in the hemopoietic organs (with exception of yolk sac) were reported to form clusters with erythroblasts [4], these macrophages might contribute to the erythrocyte differentiation. Another example is mAb SER-4, which recognizes sialic acid-specific sialoadhesin belonging to the immunoglobulin superfamily [4, 5]. SER-4 positive macrophages were reported to appear in embryos after 17-dpc when myelopoiesis starts [6].

Mouse macrophage galactose/*N*-acetylgalactosamine-specific calcium-type lectin (MMGL), one of macrophage endogenous lectins, was previously characterized [7–10].

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MMGL is a 42 kDa type II transmembrane glycoprotein with single carbohydrate recognition domain in its C-terminus [8]. The lectin is originally described to be specific for galactose and *N*-acetylgalactosamine, it is shown to bind specific oligosaccharides and glycopeptides [9]. In adult mice, our previous investigations revealed that the expression of MMGL is restricted to macrophages localized in the connective tissue and in the interstitial tissue, typically dermis and subcutaneous layer of skin as well as perivascular connective tissue [10]. In the case of lung, MMGL-positive cells were restricted to the connective tissue surrounding blood vessels and respiratory epithelia, whereas alveolar macrophages did not express this molecule [11]. Detection of MMGL and its localization in mouse embryos at various developmental stages is one of the most fundamental questions to understand functions of MMGL during embryogenesis. Thus, we asked when and where MMGL-positive macrophages appeared in mouse embryonic tissue. In this communication, we describe a survey for the expression of MMGL in mouse embryos by means of biochemical and immunohistochemical methods using MMGL-specific mAbs that we previously developed [12].

Materials and methods

Mice

Specific pathogen-free (SPF), pregnant and adult CD-1 (ICR) mice were purchased from SLC (Japan).

Cells

Rat hybridoma cell lines producing mAb against MMGL (LOM-14, $IgG2_b$; LOM-4.7, $IgG2_a$) [12], against CD45 (200 kDa) (M1/9.3.4.HL.2, $IgG2_a$; American Type Culture Collection (ATCC), USA) were cultured in Dulbecco's modified Eagle medium (D-MEM; Nissui Pharmaceutical, Japan) containing 4.5 g l^{-1} glucose, 10% heat inactivated fetal bovine serum (FBS; Bioproducts, Inc., MD, USA), 100 U ml^{-1} penicillin (Wako Pure Chemicals, Japan) and 100 μg ml^{-1} streptomycin (Sigma Chemical Co., USA) in humidified atmosphere with 5% CO_2; 95% air at 37 °C. A rat hybridoma cell line F4/80 ($IgG2_b$; ATCC) was cultured in RPMI 1640 (Gibco BRL; USA) supplemented with 10% FBS and penicillin/streptomycin as for D-MEM. Hybridoma culture supernatants were used as a source of mAb.

Detergent extraction and affinity chromatography

Embryos, removed from anesthetized pregnant ICR mice, were homogenized in a lysis buffer (1% Triton X-100, 20 mM 3-[*N*-morpholino] propanesulfonic acid [MOPS], 150 mM NaCl, 20 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.02% NaN_3, pH 7.0) containing 0.1 μM aprotinin (Sigma), 1 μM pepstatin A (Sigma), 1 μM leupeptin (Sigma) and 1 mM phenyl methyl sulfonyl fluoride (PMSF, Sigma) using Potter-Elvehjem homogenizer, then extracted for 1 h on ice (1 ml lysis buffer per 100 mg embryo wet weight). The homogenates were centrifuged at 100 000 × **g** for 30 min and the supernatants were collected. Protein concentration in the embryo lysates was assessed using a BCA protein assay kit (Pierce, USA) and the protein concentration was adjusted to 3.3 mg ml^{-1} in the lysis buffer. Galactose-Sepharose beads were prepared using Sepharose 4B (Pharmacia Biotech, Sweden) according to the published procedures [13, 14]. Three milliliter of each embryo lysates (total 10 mg protein) were added to a 1 ml packed volume of galactose-Sepharose beads and the suspension was continuously mixed for 18 h at 4 °C on a rotater. The suspension was transferred into a small plastic column ($\phi 7.5 \times 60$ mm) and was washed with 20 ml of 0.1% Triton X-100, 20 mM MOPS, 500 mM NaCl, 20 mM $CaCl_2$ and 0.02% NaN_3 (pH 7.0). The bound materials were eluted with 10 mM EDTA, 0.1% Triton X-100, 20 mM MOPS, 500 mM NaCl and 0.02% NaN_3 (pH 7.0) by collecting 1 ml aliquots of fractions. In these experimental conditions, the bound materials were recovered in the second fraction and an aliquot of this fraction was analysed for the presence of MMGL.

Electrophoresis and Immunoblotting

A 6 μl aliquot of each sample in the eluted fraction from galactose-Sepharose column was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% gel, non-reducing conditions) according to the method of Laemmli [15]. The proteins in the gel were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, USA) using Milli Blot-SDE system (Millipore). The membrane was treated in phosphate-buffered saline (PBS) containing 5% normal goat serum and 3% bovine serum albumin (BSA; Seikagaku Corp., Japan) for 18 h at 4 °C to block nonspecific binding sites. The membrane was incubated with mAb LOM-14 (1 : 2 dilution in PBS containing 0.1% Tween-20 [PBS-Tween]) for 90 min at room temperature, followed by an incubation with horseradish peroxidase (HRP)-conjugated goat anti-rat IgG(H + L) (Zymed, USA) diluted at 1 : 1000 in PBS-Tween for 90 min at room temperature. Signals for MMGL were visualized using ECL Western blotting detection reagent and Hyper-film ECL (Amersham, UK).

Immunohistochemistry for light microscopy

Whole embryos were embedded in O.C.T. compound (Miles, USA) and frozen. Sections (10 μm thick) were made by cryostat, picked up on poly-L-lysine (Sigma)-coated slides and were fixed in ice cold acetone for 30 s. Endogenous peroxidase activity was blocked by an incubation with 1% periodate for 10 min. Nonspecific binding sites were blocked using 5% normal mouse serum and 3% BSA in Dulbecco's PBS (DPBS; containing 0.91 mM $CaCl_2$, 0.49 mM $MgCl_2$) for 30 min at room temperature. The sections were incubated with a culture supernatant containing

mAb for 60 min and then with biotinylated anti-rat κ and λ (Sigma) (1 : 100 dilution in 1% BSA/DPBS) for 30 min. Then the sections were incubated with HRP-streptavidin (Zymed) (1 : 100 dilution in 1% BSA/DPBS) for 30 min. The antibody binding was histochemically detected using AEC kit (aminoethyl carbazole; Zymed) supplemented with 10 mM NaN_3 to block endogenous peroxidase activity. After color development, the sections were postfixed with 2.5% glutaraldehyde in DPBS for 30 min, counterstained in Mayer's Hematoxylin, mounted with an aqueous mounting solution (Immu-mount; Shandon Inc., USA), and then observed under a light microscope and photographed. Cytochemical control was made by reaction with 1% BSA/DPBS instead of primary antibody.

Immunohistochemistry for electron microscopy

Trunk region of 14-dpc embryos was prefixed with 2% formaldehyde in DPBS for 5 min and frozen in O.C.T. compound. Sections were made by cryostat, picked up on poly-L-lysine-coated slides, and blocked with 5% normal rabbit serum and 3% BSA/DPBS. The sections were incubated with mAb LOM-14 (culture supernatant) for 18 h at 4 °C. After an additional fixation with 4% formaldehyde/DPBS for 30 min, the sections were incubated with HRP-conjugated rabbit anti-rat IgG(H + L) (Jackson Immuno Research Inc., USA) (1 : 50 dilution in 1% BSA/DPBS) for 1 h at room temperature. The antibody binding was detected by treatment with 0.05% diaminobenzidine (Sigma), 0.005% H_2O_2 in DPBS for 20 min. The sections were postfixed with 2.5% glutaraldehyde and treated with 1% osmium tetroxide (PGM Chemicals Ltd, Germany) for 30 min. The specimens were embedded in Epon 812 (TAAB Laboratories Equipment Ltd, Sweden), processed for electron microscopy and observed with an electron microscope (JEM-1200EX; JEOL, Japan) as described previously [16]. Cytochemical control was made by reaction with 1% BSA/DPBS instead of primary antibody.

Results

Biochemical analysis of MMGL expression in mouse embryos during development

To ensure quantitative comparison of MMGL in various stages of embryos [17] as much as possible, a fixed ratio of lysis buffer and wet weight of embryos was maintained during extraction. An equal amount of the embryo extract (protein basis) was mixed with an equal volume of galactose-Sepharose beads in the presence of calcium to adsorb active lectins. The materials eluted from the beads with EDTA were analysed by immunoblotting using mAb LOM-14. We have already demonstrated the lack of cross reactivity of mAb LOM-14 to mouse hepatic lectins and appropriateness of the method based on a combination of affinity chromatography and immunoblot analysis for quantitation of MMGL [10]. A 42 kDa band representing the signal for MMGL was first detected in embryos at 11-dpc (Figure 1). We could not detect significant signals for MMGL in 10-dpc embryos in an independent experiment (data not shown). At 12-dpc embryos, the signal for MMGL was dramatically increased, and another major increase was observed at 14-dpc embryos. The signal level for MMGL was maintained, as far as we examined, until 18-dpc embryos.

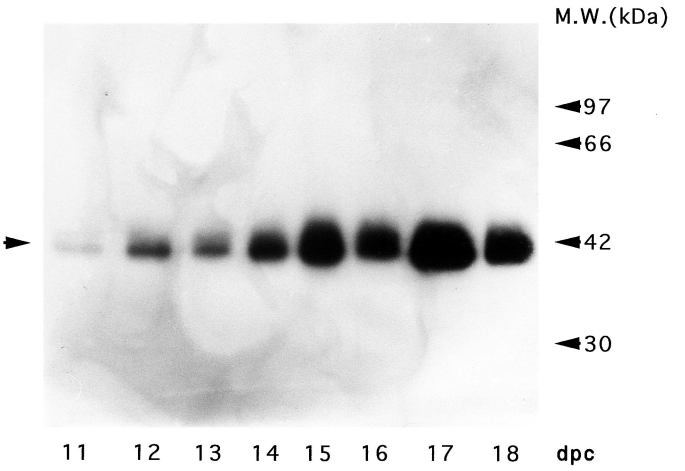
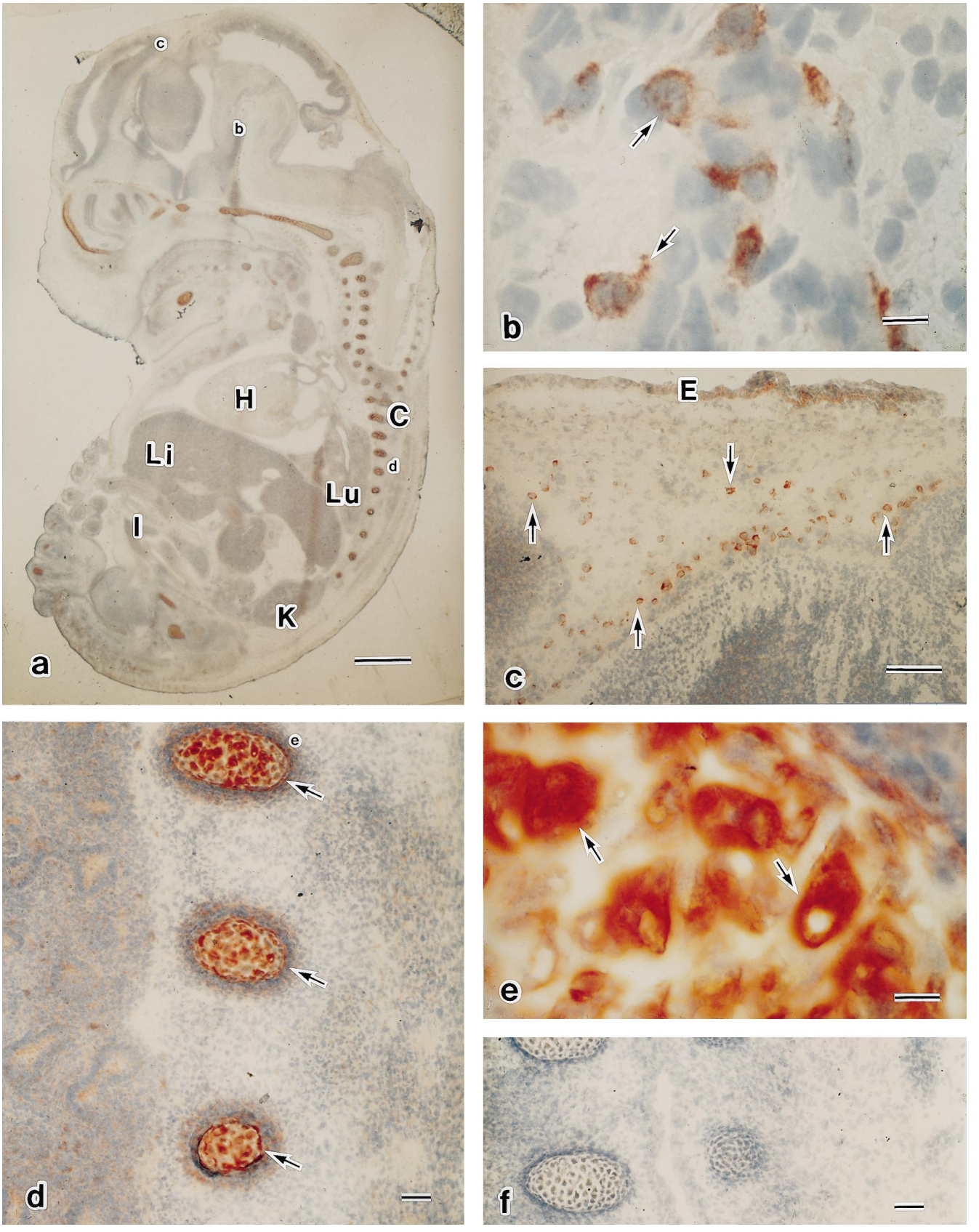


Figure 1. Immunoblot analysis for MMGL in detergent lysates of mouse embryos at various developmental stages. Whole embryos were extracted with Triton X-100, and an aliquot was applied on a galactose-Sepharose column. The bound materials were eluted with EDTA. Each eluate from the column was electrophoretically separated by SDS-PAGE under non-reducing conditions, transferred to a PVDF membrane. The membrane was stained using mAb LOM-14 and HRP-goat anti-rat IgG(H + L), and the signals for MMGL were visualized using the ECL system. Numbers shown under each lane indicate embryonic stages in days post coitus (dpc). Molecular weight markers are phosphorylase b (97 kDa), BSA (66 kDa), aldolase (42 kDa) and carbonic anhydrase (30 kDa). The arrow indicates the position of MMGL.

Immunohistochemical analysis of MMGL expression in mouse embryos by light microscopy

We next examined localization of MMGL-positive cells within mouse embryo sections. We have examined 12, 14, 16, 18-dpc embryos [17], newborn and 6-week-old adult mice.

A representative sagittal section of 14-dpc embryos is shown in Figure 2a. In 14-dpc embryos, MMGL-positive cells were typically observed in mesenchyme. These include mesenchyme of the midbrain region (Figure 2b), beneath the epidermis (Figure 2c), the under part of jaw and around the intestine under organ development. The marginal layer of spinal cord and the choroid plexus of fourth ventricle also contained MMGL-positive cells. Fetal liver, which performs hemopoiesis and produces monocytes/macrophages at this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stage, did not exhibit positive signals. MMGL-positive cells were not typically detected within the interstitial tissue of developing major organs, such as heart, lungs, intestine and kidney. In 12-dpc embryos, positive staining for MMGL was detected in mesenchymal cells localized beneath embryonic skin, though the frequency of positive cells was low at this stage (data not shown). As developmental stage advanced (from 16 to 18-dpc embryos), the mesenchymal cells expressing MMGL were increased in number in the region beneath embryonic skin (data not shown).

Transient expression of MMGL in intermediate cartilage tissue

A conspicuous signal for MMGL was observed in an intermediate cartilage tissue, where embryonic mesenchymal cells condense and differentiate into chondroblasts. Typical results at 14-dpc embryos were demonstrated (Figure 2a and d). Chondroblasts in the transient cartilage during endochondral ossification process were intensely stained with mAb LOM-14 (Figure 2e).

The signals for MMGL from chondroblasts were seen for every site under the process of endochondral ossification as far as we observed. Cytochemical control did not produce such a signal (Figure 2f). Another mAb LOM-4.7, which is specific for MMGL and recognizes an independent epitope on MMGL [12], also stained the chondroblasts (data not shown). As controls, a mAb specific for F4/80 antigen (a macrophage marker) and a mAb specific for CD45 (leukocyte common antigen) did not stain the chondroblasts (data not shown).

The distribution of MMGL-positive cells and the level of signals for MMGL in chondroblasts changed during the course of embryonic development. In 12-dpc embryos, MMGL-positive cells were detected beneath the epidermis as well as in the transient cartilage for developing femur (data not shown), which is under condensation. In 16-dpc embryos, the number of MMGL-positive cells in mesenchyme beneath epidermis was increased, whereas the number of MMGL-positive chondroblasts was decreased. Furthermore, the signal intensity for MMGL in these chondroblasts was also decreased as calcification proceeded. The calcified area within the intermediate cartilage is devoid of MMGL-positive cells. In 18-dpc embryos and newborn mice, the number of MMGL-positive cells distributed in the dermal connective tissue was increasing, whereas chondroblasts were scarcely MMGL-positive.

In adult mice, the xiphoid process and the cartilage of auricle were examined for MMGL expression as examples of hyaline and elastic cartilage, respectively. Chondroblasts and chondrocytes in these regions were devoid of staining (data not shown).

Electron microscopic detection of MMGL in chondroblast

In 14-dpc embryos, electron microscopic studies of chondroblasts in the vertebral body revealed a positive reaction for MMGL primarily in the rough endoplasmic reticulum (Figure 3).

Discussion

Production of macrophage-specific monoclonal antibodies has contributed to studies on ontogeny of macrophage during development. In the case of mouse embryos, two types of mAbs have been mainly utilized for this purpose, F4/80 [3] and SER-4 (sialoadehesin) [5, 6]. The first appearance of cells bearing F4/80 antigen, a mature macrophage marker, has been reported in yolk sac and fetal liver in 10-dpc embryos [3]. In contrast, the appearance of SER-4 positive cells was reported to occur at a much later stage of embryogenesis in developing lymphohematopoietic organ from about 17-dpc embryos in association with the beginning of myelopoiesis [6]. Since SER-4 defines sialoadhesin, which is a sialic-acid binding cell surface receptor, macrophage recognition of sialic acid-containing glycoconjugates through this receptor may not be required until this stage. In the present study, we detected cells expressing MMGL as early as 11-dpc embryos by immunoblotting analysis. In addition, immunohistochemical study revealed the presence of MMGL-positive cells as early as 12-dpc embryos. The early appearance of this carbohydrate recognition receptor, which is a galactose/*N*-acetylgalactosamine-specific calcium-type lectin, is in contrast to the late appearance of sialoadhesin. This may suggest the requirement of carbohydrate

Figure 2. Light micrographs to show immunohistochemical localization of MMGL in 14-dpc embryos. Tissue sections were incubated with mAb LOM-14, and the bound antibodies were visualized using biotinylated anti-κ/λ plus HRP-streptavidin (brown). Nuclei were stained with Mayer's Hematoxylin (blue purple). (a) A sagittal section of 14-dpc embryos. H, heart; Li, liver; I, intestine; C, vertebral bodies; Lu, lung; K, kidney. The bar represents 1 mm. (b) Higher magnification of the area indicated by small letter b in Figure 2a. Midbrain region. Arrows indicate MMGL-positive cells. The bar represents 10 μm. (c) Higher magnification of the area indicated by small letter c in Figure 2a. Mesenchyme beneath epidermis of the top of head. Arrows indicate MMGL-positive cells. E: epidermis. The bar represents 100 μm. (d) Higher magnification of the area indicated by small letter d in Figure 2a. All chondroblasts in the vertebral bodies (arrows) are stained with mAb LOM-14. The bar represents 50 μm. (e) Higher magnification of the area indicated by small letter e in Figure 2d. Chondroblasts (arrows) in the intermediate cartilage tissue of vertebral body. Positive reaction was detected in cytoplasm. The bar represents 10 μm. (f) Cytochemical control for the intermediate cartilage tissue. Positive staining was not observed. The bar represents 50 μm.

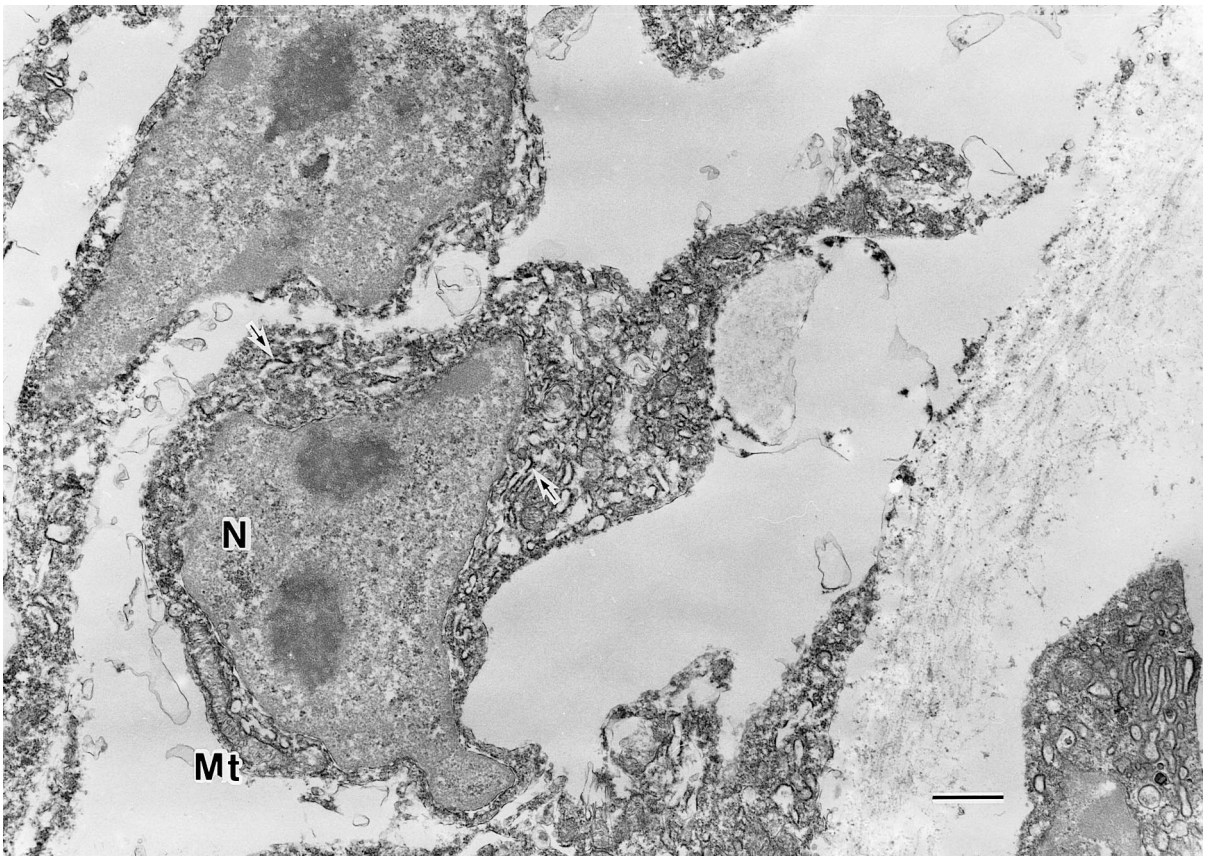

Figure 3. Ultrastructural localization of MMGL in chondroblasts in the vertebral body (14-dpc embryos), was immunohistochemically detected using mAb LOM-14 and HRP-labeled second antibody. Arrows indicate positive reaction representing MMGL in rough endoplasmic reticulum. N, nucleus; Mt, mitochondria. The bar represents 1 μm.

recognition based on galactose or *N*-acetylgalactosamine by macrophages from a relatively early stage of fetal development.

During the stages between 12 and 18dpc, MMGL-positive mesenchymal cells were distributed throughout the embryonic body (Figure 2), but the major site where these cells were abundant was the region beneath the epidermis throughout this period. The distribution of MMGL-positive cells was quite different from that of F4/80-positive cells at this stage. F4/80-positive cells were reported to be abundant in fetal liver after 10-dpc, and the number of F4/80-positive cells in fetal liver was reported to reach at peak levels at 15-dpc mainly in erythropoietic islands [2]. The absence of MMGL-positive cells in fetal liver despite the abundance of F4/80-positive macrophages during this stage is a characteristic feature of this marker.

In our previous study, we reported a restricted distribution of MMGL-positive macrophages in the connective tissue surrounding blood vessels and respiratory epithelia in adult mouse lungs in contrast to the absence of MMGL in alveolar macrophages [11]. Such connective tissue specificity in adult mice was not a lung specific phenomenon but was seen in a wide variety of tissues and organs [10]. That is, MMGL-positive cells were abundant in the dermis as well as in the interstitial tissue of a variety of organs including lungs, heart, skeletal muscles, gastrointestinal tract, urinary bladder and thymus. In contrast, the interstitial tissue of developing embryonic organs, which includes heart, lungs and intestine, was essentially negative for the expression of MMGL at least until 18-dpc embryos. These results suggest the presence of MMGL-positive macrophages in the connective tissue or interstitial tissue seen in all adult mouse tissue except for skin, is established in a relatively later stage of embryonic or postnatal development.

One of the most interesting observations in this study is the immunohistochemical detection of the transient expression of MMGL in embryonic cartilage tissue during endochondral ossification process. Light and electron microscopic observations indicated that cells with MMGL expression were not macrophages but chondroblasts. The binding of anti-MMGL mAb (mAb LOM-14) to embryonic chondroblasts is not likely to be due to a fortuitous cross

reaction. There are proteoglycans as an extra cellular matrix protein in mature cartilage as well as cartilage under development. Cartilage proteoglycans (aggrecan, PG-H) and fibroblast proteoglycans (versican, PG-M) are known to contain a calcium-type lectin domain on their C-terminal regions. However, since the molecular weight of these proteoglycans are very high (180–370 kDa for core protein and 2500 kDa for mature proteoglycan in the case of rat aggrecan [18], 300–550 kDa for core protein in the case of chicken versican [19]), it is clearly distinguishable from MMGL (42 kDa) by immunoblot analysis. Furthermore, amino acid sequences of the lectin domain of mouse aggrecan [20] and mouse versican [21] are not closely related to the sequence of lectin domain in MMGL. In addition, the lectin domain of rat aggrecan with galactose/fucose specificity [22] is not closely related to that of rat MMGL homologue with galactose/*N*-acetylgalactosamine specificity [23]. In contrast, our previous results [10] showing that mAb LOM-14 did not exhibit cross reactivity to mouse hepatic lectins, which are more closely related to MMGL than they are to the lectin domains of aggrecan and versican, indicate that cross reaction of mAb LOM-14 to the proteoglycans is less likely. Furthermore, the signals representing MMGL were not generally detected in cartilage. Thus, they were only detected in the transient cartilage tissue in mouse embryos but were absent from adult mouse cartilage. Finally, binding of mAb LOM-4.7, which recognizes an independent epitope on MMGL, to chondroblasts in the transient cartilage tissue also supports the existence of an MMGL molecule.

The MMGL expression in chondroblasts appears to be restricted to the chondroblasts in an early stage of bone development such as chondroblast condensation stage as well as at the stage immediately after their condensation. The signal intensity for MMGL in the transient cartilage tissue peaked in 14-dpc embryos. The level of MMGL expression was decreased in the chondrocytes with hypertrophy, suggesting that MMGL expression decreases as the maturation process of chondroblasts advances. It is also supported by the fact that MMGL expression disappeared in the area with calcification.

It is too early to speculate functions of MMGL in embryonic chondroblasts. The absence in adult cartilage may suggest that it is not involved in the cartilage function in general. The transient nature of its expression may suggest that it is a developmentally regulated phenomenon contributing to certain processes of bone development by some unknown way. Electron microscopic studies revealed the presence of MMGL signals in rough endoplasmic reticulum. This result demonstrates a *de novo* synthesis of MMGL in the chondroblasts, suggesting that MMGL produced by chondroblasts may actively contribute to the developmental processes. Recently, we have cloned the human counter part of MMGL, human macrophage lectin (HML) [24]. Monoclonal antibodies specific for recombinant HML showed a similar distribution in human tissues [25]. Most interestingly, one of these antibodies was shown to react with adult cartilage (Sano *et al*., unpublished observation). Differences in the distribution and the molecular functions of MMGL and HML remains to be established.

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