Ultrastructural Localization of a Macrophage-Restricted Sialic Acid Binding Hemagglutinin, SER, in Macrophage-Hematopoietic Cell Clusters

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Resident bone marrow macrophages in hematopoietic clusters have previously been shown to express a novel lectin-like sheep erythrocyte receptor, SER, which mediates binding of unopsonized sheep erythrocytes via recognition of sialylated glycoconjugates and may interact with sialylated ligands on murine bone marrow cells. In this study, the distribution of SER on macrophages within hematopoietic clusters was localized by a monoclonal antibody, SER-4, by immunofluorescence and immunoelectron microscopy. SER was found to be diffusely localized at the contact zones between macrophages and erythroblasts, whereas the receptor was highly concentrated at the contacts between macrophages and developing myelomonocytic cells. These data suggest that SER on resident bone marrow macrophages interacts differentially with sialylated ligands on developing myeloid cells and that this may influence their development.

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MATERIALS AND METHODS

Antibodies. Monoclonal antibody SER-4, a rat immunoglobulin (Ig) G2a directed to SER on mouse macrophages, was used as a tissue culture supernatant in immunofluorescence experiments and in the form of biotinylated IgG in immunoelectron microscopy experiments. F4/80, a rat IgG2b anti-mouse macrophage monoclonal antibody that recognizes a distinct plasma membrane glycoprotein, was used as an 18% sodium sulphate precipitate of tissue culture supernatant. Biotinylated sheep IgG anti-rat IgG was from Cappel Laboratories (Cochranville, PA); streptavidin-Texas Red was purchased from Amersham Corp (Arlington Heights, IL); and streptavidin-peroxidase (Cappel) and streptavidin-gold were from Janssen Pharmaceutical (Beerse, Belgium). All antibodies were used at saturation.

Isolation of bone marrow clusters. Isolation of bone marrow clusters was performed as described in detail with modifications. Briefly, the femoral bone marrow plugs from six CD1 adult male mice were digested for 30 minutes at room temperature with 30 mL 0.05% collagenase in Dulbecco’s medium. To purify the resulting clusters from the cell suspension, 5-mL portions of the digest were layered over Percoll gradients (Pharmacia LKB, Ltd, Uppsala, Sweden) prepared as follows: 76 mL isotonic Percoll (9:1 Percoll: 10X phosphate-buffered saline (PBS), Dulbecco A + B was mixed with 105 mL Dulbecco’s medium (GIBCO, Ltd, Paisley, Scotland), 5 mL 1M Hepes buffer (GIBCO), pH 7.4, and 5 mL fetal calf serum (FCS, Seromed, Berlin, FRG). The mixture was divided into 6 x 34 mL tubes and gradients were performed by centrifugation at 18,000 rpm for 20 minutes at 4°C in a Sorvall centrifuge using a SS34 fixed angle rotor. Gradients were stored on ice for up to 2 hours before use. After layering the digest, the gradients were centrifuged in a refrigerated benchtop centrifuge at 50g for 15 minutes at 4°C. The lower bands containing pure clusters were removed and the clusters were washed twice in Dulbecco’s medium by centrifugation at 100g for 10 minutes at 4°C.

For morphologic analysis by electron microscopy, purified clusters were fixed in suspension for 10 minutes at room temperature using 4% glutaraldehyde- 1. Lysine buffer, as described previously. For immunofluorescence and immunoelectron microscopy, clusters were suspended in Dulbecco’s medium containing 10% FCS and plated on 13-mm diameter glass coverslips or 35-mm diameter tissue culture-treated plastic Petri dishes, respectively. After 2 to 3 hours at 37°C

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in the presence of 5% CO₂ more than 90% of the clusters had become firmly attached to the substrate via the central macrophages.²

Immunofluorescence. Live adherent clusters on glass coverslips were incubated on ice with or without first antibody, either SER-4 conditioned medium, diluted 1:5, or F4/80 sodium sulphate precipitate, diluted 1:20, in Dulbecco’s medium containing 1% FCS, 20 mmol/L Hepes, and 10 mmol/L sodium azide. After 1 hour, coverslips were rinsed five times in ice-cold Dulbecco’s medium and fixed for 30 minutes at 4°C in 4% paraformaldehyde, freshly prepared in PBS. All subsequent procedures were at room temperature and all dilutions were in PBS. The fixed clusters were quenched for 15 minutes in 50 mmol/L ammonium chloride and nonspecific binding sites blocked by sequential incubation for 30 minutes in 1% Marvel skimmed milk produced by Cadbury’s and 1% normal sheep serum. Coverslips were then incubated for 60 minutes with biotinylated sheep IgG anti-rat IgG, diluted 1:100. After washing for 10 minutes, coverslips were mounted under water and visualized by phase-contrast or fluorescence microscopy using a Zeiss Photomicroscope III.

Immunoelectron microscopy. Live adherent clusters on 35-mm plastic Petri dishes were incubated for 60 minutes on ice with 2 μg/mL biotinylated SER-4 IgG, diluted in Dulbecco’s medium containing 1% FCS, 20 mmol/L Hepes, and 10 mmol/L sodium azide. Clusters were washed in ice-cold Dulbecco’s medium and fixed for 30 minutes in Nakane’s fixative at 4°C.¹³ After quenching for 30 minutes with 50 mmol/L ammonium chloride and blocking nonspecific binding sites with 1% Marvel, the clusters were incubated in streptavidin-peroxidase for 1 hour at room temperature. Dishes were washed in PBS, then postfixed with 1.5% glutaraldehyde in 0.1 mol/L sodium cacodylate containing 1% sucrose, pH 7.4. They were subsequently processed for the demonstration of peroxidase as previously described.¹³ As a control for nonspecific binding of biotinylated SER-4 IgG, clusters on Lab-Tek (Nunc, Naperville, IL) slides were stained in parallel, in the presence or absence of a 50-fold excess of unlabeled SER-4 IgG. After the reaction with diaminobenzidine, the clusters were examined for reaction product by light microscopy.

RESULTS

The distribution of SER-4 antigen on resident bone marrow macrophages within isolated hematopoietic clusters was first analyzed by immunofluorescence; after optimization of staining conditions, the experiments were extended to analysis of fine structure.

Immunofluorescence. The central macrophages of isolated bone marrow clusters spread extensively on glass, retaining the hematopoietic cells exclusively attached to the upper surfaces of their plasma membrane (Fig 1A), as previously described.²⁵ Two patterns of immunofluorescence were seen after staining with the SER-4 monoclonal antibody. Approximately 25% of the clusters contained macrophages that showed a uniform pattern of plasma membrane immunofluorescence (not shown). However, the majority of clusters showed an uneven pattern of immunofluorescence, with bright labeling of macrophages at their contact points with a subpopulation of the attached hematopoietic cells (Fig 1B). The intensity of the fluorescence “caps” was heterogeneous, but examination of this subpopulation by phase-contrast microscopy demonstrated that they consistently had the morphology of myelomonocytic precursor cells (Fig 1A). In contrast, the attached erythroid cells exhibited a more diffuse fluorescence at the contact zones with macrophages (Figs 1A and B). These findings were reproduced in three independent experiments by immunofluorescence and in two experiments by immunoperoxidase labeling. On all occasions, intense labeling of the SER-4 antigen was restricted to the contact points of macrophages with cells bearing the morphology of myelomonocytic precursors.

Parallel staining with the macrophage-restricted monoclonal antibody F4/80 consistently revealed a uniform pattern of immunofluorescence on the macrophage plasma membrane of all clusters, as observed previously by immunoperoxidase labeling.²⁵ This control demonstrates that the immunofluorescence pattern obtained with SER-4 was not an artefact of the staining method.

Morphology of clusters examined by electron microscopy. Initially, we examined the morphology of macrophage clusters by electron microscopy, prior to adherence and without immunoperoxidase staining (Figs 2 and 3). The clusters were frequently composed of several adherent macrophages (Figs 2 and 3B) as well as a more fibroblastic type of stromal cell (Fig 3A). Mixed populations of developing erythroblasts in various stages of maturation and immature granulocytes were adherent to the plasma membrane of single macrophages. Cells in mitosis were frequently seen, still adherent to the macrophage (Fig inset 2). The fibroblast-type of stromal cell was also present in clusters, but in fewer numbers. Its long arms enveloped developing granulocytes as well as erythroblasts (Fig 3A).

Immunoperoxidase staining examined by electron microscopy. Following immunoperoxidase labeling of adherent clusters with SER-4, we frequently observed a high concentration of peroxidase reaction product at the contact zones of neutrophilic (Fig 4A) and eosinophilic (Fig 4B) granulocytes. In contrast, at the erythroid-macrophage contact zones, the antigen was consistently found to be more diffusely localized (Fig 5A). We observed a single macrophage pseudopodium extending between a granulocyte and a erythroblast in which the reaction product was concentrated only on the plasma membrane in contact with the granulocyte (Fig 5B). It should be noted that bleeding of peroxidase reaction product also occurred and the labeling sometimes observed on adjacent cells (Fig 5A) is an artefact of the immunoperoxidase procedure, as described by others.¹² No labeling was seen in control preparations in which unlabeled SER-4 monoclonal antibody was added in 50-fold excess to the biotinylated SER-4.

Thus, it would appear that the “capped” antigen seen by immunofluorescence in Fig 1 corresponds to the heavier, more restricted distribution of SER-4 antigen seen in Figs 4 and 5 and, in both cases, is present selectively at the zones of contact between macrophages and developing granulocytes.

DISCUSSION

In the present study, we have observed two distinct localization patterns for the SER antigen in macrophage-hematopoietic clusters isolated from bone marrow. SER was diffusely expressed at the contact sites of RBMM with developing erythroid cells but was selectively concentrated at the contacts with myelomonocytic precursors. This finding provides indirect evidence that SER interacts with a ligand.
on the myeloid subpopulation of attached hematopoietic cells. Similar types of observation have been made with other receptor-ligand interactions. For example, certain cell adhesion molecules, such as the fibronectin receptor, become concentrated in adhesion plaques at the sites of contact with appropriate substrates. In the case of the leukocyte integrin, CR3, its ligand, iC3b, must be clustered within the membrane to mediate high avidity binding and phagocytosis. Capping of receptors with ligand may also occur with cell surface receptors whose primary function is not necessarily related to cellular adhesion but rather to cellular signaling. Thus, on T lymphocytes, the accessory molecule CD4 has been shown to become concentrated along with the T cell receptor at the contact points formed with antigen-presenting cells. This is thought to be due, at least in part, to the mutual capping of CD4 with its ligand, major histocompatibility complex class II antigen, on antigen-presenting cells.

The selective association of “capped” SER with myeloid cells was initially indicated by phase contrast microscopy in conjunction with immunofluorescence and was then confirmed unambiguously by immunoelectron microscopy. This selectivity of SER localization is explained most simply by postulating that myeloid cells express on their surface a higher concentration of the ligand recognized by SER than do erythroid cells. Assuming that SER is free to diffuse laterally within the plasma membrane, this would be expected to lead to a higher concentration of the ligand recognized by SER at the contact points of myeloid cells, as observed in the present study. The heterogeneity in the intensity of fluorescence, which was observed among the attached myeloid cells, may
Fig 2. Electron micrographs of isolated hematopoietic clusters before adherence. Two bone marrow macrophages (M) with obvious endocytosed material can be seen clinging together. One of the macrophages has a number of developing erythroblasts (E) at various stages of maturation and one plasma cell (P). (Original magnification × 10,000.) (Inset) An erythroblast (E) can be seen in mitosis, while still adherent to the macrophage (M). (Original Magnification × 16,000.)
Fig 3. (A) In this preparation, a portion of another type of stromal cell is shown. This cell has very condensed cytoplasm and its plasma membrane is closely associated with extracellular fine filaments (f). It also has long processes (arrows) extending to the adjacent developing granulocytes (G) and erythroblasts (E). (Original magnification ×10,000.) (B) A higher magnification of the contact zones (arrowheads) between promyelocytes (G), erythroblasts (E), and the macrophage (M) plasma membrane. (Original magnification ×17,000.)
Fig 4. An immunoperoxidase procedure is used to demonstrate the localization of the SER-4 antibody through the localization of reaction product. (A) Note the concentration of reaction product in the contact zone (arrowheads) between two developing granulocytes (G) and the macrophage (M) membrane. (Original magnification × 16,000.) (B) High levels of reaction product were also found in the contact zones (arrowheads) between developing eosinophils (Eos) and the macrophage (M) membranes. (Original magnification × 25,000.)

be due to variable surface expression of SER ligands, which in turn could be related to their stage of differentiation. An alternative explanation is that ligands for SER are expressed at similar levels on all hematopoietic cell subpopulations, but that in erythroid cells the cytoskeleton selectively retards lateral mobility within the plasma membrane, thus preventing the stable formation of a “cap” at the points of cell contact.\textsuperscript{19,20} Using cultured SER + peritoneal macrophages, we have recently found that following the addition of single cell suspensions of total bone marrow cells, the immature
myeloid subpopulation binds preferentially to SER (P. Crocker, unpublished observations). This supports the possibility that myeloid cells express higher levels of ligand for SER. We are currently exploring the nature of the ligand(s) on hematopoietic cells recognized by SER.

Despite the in vitro capacity of SER to mediate binding of hematopoietic cells, its primary function on RBMMs in hematopoietic clusters is unlikely to be as a cell adhesion molecule. Reversible, high-avidity adhesion of myeloid and erythroid cells to RBMMs is mediated by a calcium-
dependent receptor, designated EbR. Recent experiments have shown that monoclonal and polyclonal antibodies directed to SER do not affect binding of hematopoietic cells to RBMMs in the presence of divalent cations (L. Morris, P. Crocker, and S. Gordon, manuscript in preparation). It is likely, therefore, that the attachment of hematopoietic cells to RBMMs is primarily via EbR, and that this then allows SER to interact with appropriate sialylated glycoconjugates on the attached cells.

In addition to the bone marrow, SER is expressed at high levels on lymphoid stromal macrophages that are in close proximity to mature B cells, notably, within the marginal zone of the spleen and the subcapsular and medullary regions of lymph nodes. This raises the possibility that SER interacts with appropriate sialylated ligands on B lymphocyte subpopulations. In preliminary immunofluorescence experiments, however, we have failed to see a similar "capping" phenomenon on macrophage-B lymphocyte clusters isolated from the spleen (P. Crocker, unpublished observations). In the case of lymphoid tissues, it is possible that ligands for SER are only expressed following lymphocyte activation and that the ensuing receptor-ligand interaction plays a role in regulating the humoral immune response.

Although the functional significance of SER "capping" in the bone marrow is unknown, it is of considerable interest that certain other sialic acid-binding proteins have been shown to exert potent biologic effects on developing and mature hematopoietic cells. For example, cholera toxin β subunit is able to trigger mitogenesis of thymocytes and T cells can be stimulated to proliferate in response to monoclonal antibodies against GD3 ganglioside. In addition, the purified hemagglutinin of influenza A virus is able to either stimulate the proliferation of B lymphocytes or suppress the oxidative activity of granulocytes. It is possible, therefore, that the interaction of SER in the bone marrow with sialylated ligands on hematopoietic cells delivers a signal that affects their differentiation. Future studies with inhibitory monoclonal antibodies directed to SER may help to resolve the biologic role of SER in hematopoietic development.

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