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Mast Cell Isolation from the Immature Rat Brain

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Key Words
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Abstract
Mast cells are immune cells of hematopoietic origin that circulate as precursor cells prior to migration into vascularized tissues where they mature and undergo terminal differentiation in response to different cytokines within the local environment. Mast cells are well known as important regulators of inflammatory processes in peripheral tissues and recent studies support the involvement of mast cells in mediating the inflammatory response to cerebral hypoxia-ischemia in both the neonatal and adult brain. To better study mast cell function in vivo, it is important to be able to identify their environment-specific phenotype, as well as to study their interaction with other neural cells in vitro. Previous such studies of mast cells have relied on mast cells isolated from gut or bone marrow, or on a number of mast cell lines, all of which may behave differently from brain mast cells. The purpose of this study was to develop a technique for the isolation of mast cells from neonatal rat brain and to characterize these cells following hypoxia and hypoxia-ischemia. We adapted a previously described technique of coupling an antibody to the mast cell-specific FcεR1 receptor to a MACS microbead for the selective removal of intact mast cells from a neonatal brain preparation. We have isolated toluidine blue-positive brain mast cells that provide substrate for both protein analysis and in vitro studies. These cells express proteins previously used to specifically identify microglia in the brain, Iba-1 and coronin-1a. A subpopulation of mast cells in vivo also expresses Iba-1. Thus, we report a novel method for isolation of brain mast cells suitable for the study of mast cell phenotype under a variety of conditions. Further, we suggest that the use of proteins such as Iba-1 for the identification of microglia in the brain includes the caveat that mast cells may also be detected.

Introduction

Mast cells are immune cells of hematopoietic origin that circulate as committed progenitor cells prior to migration into vascularized tissues where they mature and undergo terminal differentiation in response to different cytokines within the local environment (reviewed in Gilfillan et al. [1]). Although mast cells reside in vascularized tissues, their migration and mature phenotype appear to be controlled in a tissue-specific manner, resulting in distinct heterogeneous mast cell populations. For instance, in

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rodents and humans, mast cells from mucosal and connective tissues vary in size, function, and histamine and neutral protease content [2]. All mast cells contain cytoplasmic granules comprised of preformed mediators, such as histamine, heparin, cytokines (e.g. TNF-α), and proteases (e.g. chymase, tryptase, and matrix metalloproteases). Additionally, in response to environmental triggers that interact with a variety of cell surface receptors, mast cells can synthesize and release an array of secondary mediators, such as prostaglandins, leukotrienes, growth factors, and cytokines [3]. Thus, although mast cells were originally designated as effector cells of the innate immune response, they are now recognized as highly plastic cells capable of alterations in phenotypes that are involved in numerous aspects of health and disease [1, 4, 5].

One area in which mast cells have recently been implicated is in the response to cerebral ischemia in both adult and neonatal animals. Mast cells are normal residents in the central nervous system of both rodents and humans [6, 7], where they are found in close association with cerebral blood vessels during development and adulthood [8]. Brain mast cell numbers increase during neonatal development in the rat, coincident with the elaboration of the cerebral vasculature [8]. Dural mast cells have been shown to play a prominent role in angiogenesis [9], as well as being involved in the regulation of blood flow, opening of the blood-brain barrier, and the inflammatory response to injury [10, 11]. Recent studies from our laboratory suggest that mast cells are the 'first responders' to a hypoxic-ischemic insult in the neonatal rat [12, 13]. Hypoxia, as well as hypoxia-ischemia (HI), result in a rapid increase in brain mast cell number and state of activation/degranulation, and acute post-HI inhibition of this response with the mast cell stabilizer, sodium cromoglycate, provides long-term neuroprotection. However, the signals that initiate mast cell migration and activation have yet to be determined.

In addition, although mast cells exacerbate damage following injury to the immature rodent brain, mast cells might exhibit several phenotypes in which initially mast cells might contribute to cell death, but at later time points of recovery mast cells might facilitate tissue repair, similar to microglia and the cytotoxic M1 and neuroprotective M2 phenotypes [14]. Hence, in order to more effectively characterize the role mast cells play in neonatal brain injury and repair, it becomes necessary to isolate and characterize mast cells from the brain of the immature rodent. Currently, studies of mast cells in vitro utilize primary mast cells isolated from the pleural cavity, peritoneal cavity, bone marrow, or cultures generated from human umbilical cord blood or established mast cell lines [15, 16]. However, since the mast cell phenotype is dependent on the surrounding tissue and environmental triggers, it is critical to use mast cells derived from the same experimental paradigm.

We have adapted a methodology for the isolation of mast cells from human heart [17] for use in the immature rat brain. This isolation procedure results in a fairly pure population of brain mast cells and is applicable to both the hypoxic and hypoxic-ischemic brain. Further, we demonstrate that brain mast cells express the calcium-binding adaptor protein, Iba-1, previously considered to be a unique marker of microglia.

Materials and Methods

Animals

Timed pregnant (E15) Wistar rats (Charles River, Wilmington, Mass., USA) were purchased and allowed to deliver in individual cages. On the day of birth (P1), pups from 2–3 litters were randomized and divided into litters of 10/dam. Dams had free access to rat chow and water. All animal procedures described below were approved by the Institutional Animal Care and Use Committee of Weill Cornell Medical College and follow the NIH guidelines.

Brain Mast Cell Isolation and Cell Culture

P10–12 pups were anesthetized with isoflurane and decapitated. The brains (n = 10–20 per group) were excised, weighed, and minced with mechanical dispersion. Brain mast cells were isolated following the technique of Silver et al. [17]. Briefly, cells were centrifuged, pelleted and washed. Then, the cells were incubated with the rabbit anti-FcεR1 γ antibody followed by washing. The cells were then incubated in anti-rabbit secondary antibody conjugated to MACS beads. The cell suspension was filtered through MS-positive selection columns (Miltenyi Biotec, Bergisch Gladbach, Germany). The mast cells were counted with a hemacytometer then cultured overnight in DMEM/F12 50/50 media (Cellgro) supplemented with 10% FBS (Fisherbrand), 1% amphotericin B/penicillin/streptomycin (Quality Biological, Inc.), 100 ng/ml stem cell factor (Peprotech), and 30 ng/ml rat IL-3 (R&D Systems). The next day, the nonadherent mast cells were collected and analyzed by immunocytochemistry and Western blot. The wells were treated with trypsin and scraped to test for the presence of any adherent cells, e.g. microglia.

HMC-1 Cells

HMC-1 cells (a generous gift from the R.B. Silver lab) were maintained in cell culture with Iscove's DMEM media (Cellgro, Manassas, Va., USA) supplemented with 10% FBS (Fisherbrand), 1% amphotericin B/penicillin/streptomycin (Quality Biological, Inc., Gaithersburg, Md., USA), and 0.01% α-monothioglycerol (MP Biomedicals, Santa Ana, Calif., USA). The HMC-1 cells were used for immunocytochemistry and Western blot analysis.

Microglia

Primary microglial cultures were prepared from P2 rat pups as previously described [18]. The microglial cells were used for Western blot analysis.

Animal Model of Unilateral Cerebral HI

For isolation of brain mast cells following hypoxia, or HI, P10 rat pups of both sexes were subjected to hypoxia alone or unilateral HI according to our standard protocol and 8% O_2 for 65 min and recovery in room air [19, 20]. Pups were returned to the dams and at 24 h they were anesthetized and decapitated. Brains from the HI animals were separated into ipsilateral and contralateral hemispheres prior to subsequent processing as above for mast cell isolation. Additional animals were subjected to perfusion fixation at 48 h of reperfusion and brains processed for floating sections as previously described [12].

Immunocytochemistry

Isolated rat P9 naïve mast cells and HMC-1 cells were fixed with 4% w/v paraformaldehyde/PBS for 15 min and incubated with rabbit anti-Iba-1 (1:1,000, Wako, Osaka, Japan) for 24 h at 4°C. Next, the cells were incubated with goat anti-rabbit IgG conjugated to Alexa Fluor 594 (Jackson ImmunoResearch, West Grove, Pa., USA), avidin-FITC (1:100, Sigma-Aldrich, Steinheim, Germany), and DAPI (1:1,000, Pierce, Rockford, Ill., USA) for 1 h at room temperature. The cells were examined by fluorescence microscopy (Olympus, Center Valley, Pa., USA) with a QImaging CCD camera (QImaging, Surrey, B.C., Canada).

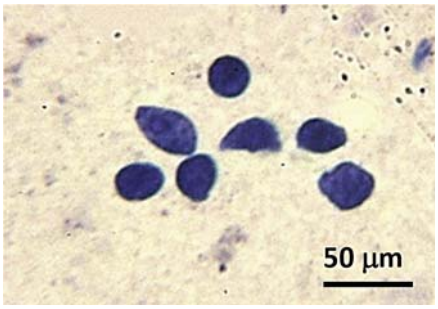
Brain sections were stained with Iba-1 (1:500, Wako) for 24 h at room temperature. Next, the sections were incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:500, Jackson ImmunoResearch) for 1 h at room temperature and counterstained with acidic toluidine blue (TB).

Western Blot

The mast cell pellet was resuspended in 100 μl of protease inhibitor buffer [50 mM Tris, pH 7.5, 1 mM EDTA, 320 mM sucrose, and 1 complete protease inhibitor cocktail tablet (Roche, Mannheim, Germany) per 10 ml buffer] and sonicated for 2 s. Total protein in each sample was determined with the Pierce BCA protein assay kit (Thermo Scientific, Rockford, Ill., USA). Samples of 10–40 μg protein were separated on a 10% SDS gel, and electrophoretically transferred onto nitrocellulose paper. Ponceau S was used to visualize the protein bands and ensure equal loading prior to Western blot analysis with the following antibodies: goat anti-c-Kit (M-14) (1:1,000; Santa Cruz, Santa Cruz, Calif., USA), goat anti-mast cell protease-1 (C-15) (1:1,000; Santa Cruz), or rabbit anti-Iba-1 (1:1,000; Wako). Each antiserum was diluted in 3% BSA. Secondary antibodies were: goat anti-rabbit (1:5,000; Jackson ImmunoResearch) or donkey anti-goat (1:5,000; Santa Cruz) diluted in PBS/5% nonfat dried milk/0.1% Triton. Bands were visualized using the Super Signal West Pico chemiluminescent substrate (Thermo Scientific) and exposed onto film.

Mass Spectrometry and Proteomic Analysis

Proteomic analysis of isolated mast cells from naïve controls, hypoxic and hypoxic-ischemic animals was conducted by the UMDNJ Neuroproteomics Core Facility, Newark, N.J., USA. Initial protein extraction was performed with 7M urea and sonication prior to separation on SDS-PAGE gels. Ten separate gel bands were excised for in-gel trypsin digestion. The resulting peptides were analyzed by liquid chromatography-tandem mass spectrometry on an Orbitrap Velos MS. The tandem mass spectrometry spectra were searched against a Uniprot rat database using both MASCOT (v.2.3) and SEQUEST search engines. A total of 3,181 proteins were identified with the protein false discovery rate less than 1%. The results were further analyzed at the Memorial Sloan-Kettering Cancer Center Proteomics Core Facility, New York, N.Y., USA.



Color version available online

Fig. 1. TB stain of freshly isolated mast cells. Mast cells were isolated from P10 rat brain as described in the Methods section. An aliquot of the final suspension was removed and stained with acidic TB for cell identification and counting. Cells are intact with densely packed vesicles.

Results

Mast Cell Isolation

Mast cells were isolated from naïve P9–10 rat brain as described in the Methods section and an aliquot was stained with acidic TB for counting. The mast cells were intact, filled with TB-positive granules and were frequently observed in clusters (fig. 1). The average yield from naïve control rat brain was approximately 5×10^5 mast cells/g wet weight brain. When mast cells were isolated from either hypoxic or hypoxic-ischemic animals, the yield was increased approximately 3-fold, to 1.6–1.7 $\times 10^6$ mast cells/g brain, although many of the TB-positive cells appeared partially to largely degranulated (not shown). This observation is consistent with our previous in vivo report that at 24 h following either hypoxia alone or HI, the mast cell number in the brain had more than doubled and the cells were variably degranulated [13].

Characterization of Isolated Mast Cells

We next characterized the isolated mast cells by Western blot analysis for c-Kit, the stem cell factor receptor expressed by all mast cells, as well as mast cell protease-1 (fig. 2a). HMC-1 cells were used as a positive control for the c-Kit protein since the antibody detects both human and rat protein. The mast cell protease-1 antibody is rat specific and detected multiple bands at 50 kDa and below in the isolated mast cells. Supporting the purity of this

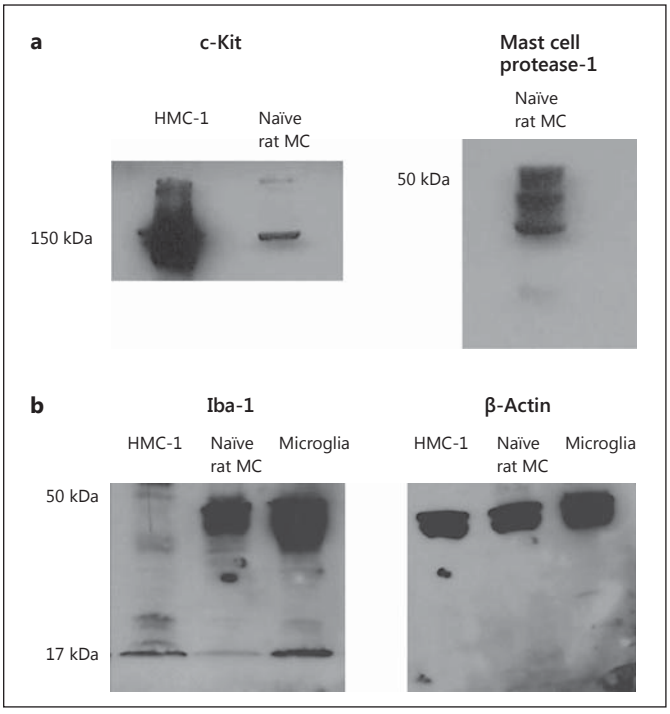


Fig. 2. Western blot analysis of isolated rat brain mast cells (MC) and HMC-1 mast cells. Whole lysates were prepared from isolated rat brain mast cells and from the human HMC-1 mast cell line and separated by SDS-PAGE. **a** c-Kit, the stem cell factor receptor, was detected as a 150-kDa protein in both HMC-1 and rat brain mast cells; HMC-1 was included as a positive control for the c-Kit antibody which recognizes both rat and human; mast cell protease-1 was detected in isolated cells using a rat-specific antibody. **b** Iba-1 was detected as a 17-kDa band in HMC-1 and rat mast cells and also as a broad 50-kDa band in the isolated rat brain mast cells. Isolated rat brain microglia was included as a positive control with bands at 17 and 50 kDa. Prominent 50-kDa bands for β-actin are seen in all 3 samples.

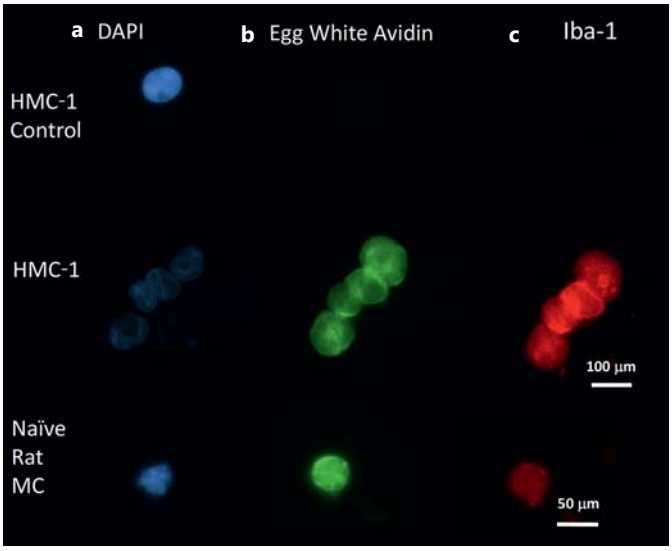


Fig. 3. Immunofluorescence for avidin and Iba-1. HMC-1 cells and isolated rat brain mast cells (MC) in culture were analyzed by immunocytochemistry. **a** Nuclear DAPI staining (blue) for intact cells. **b** Mast cell heparin sulfate granule detection with avidin (green). **c** Iba-1 staining (red). HMC-1 control cells were stained with only DAPI and the Alexa Fluor goat anti-rabbit secondary antibody.

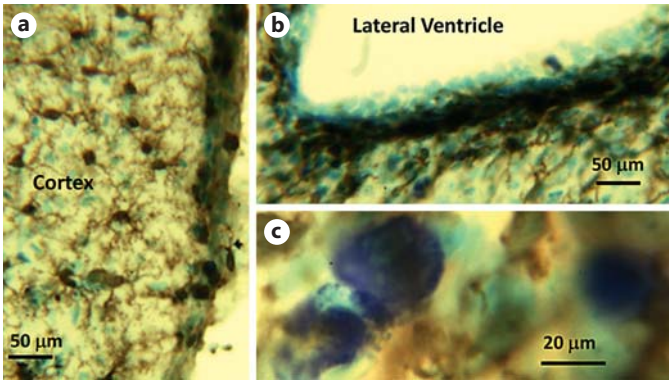


Fig. 4. TB-positive mast cells in pia express Iba-1 following HI. Rat pups were subjected to unilateral (right) cerebral HI and brains were processed 48 h later, as described in the Methods section. Free-floating sections were treated with acidic TB to label mast cells and anti-Iba-1. In addition to numerous activated, ameboid and partially ramified Iba-1-positive (brown) microglia in hypoxic-ischemic cortex (**a**) and brain adjacent to lateral ventricle (**b**), numerous TB-positive mast cells primarily in or adjacent to the pia were also Iba-1 positive. At higher magnification, the TB is more clearly visible in a representative cell from the pia lining the lateral ventricle (**c**).

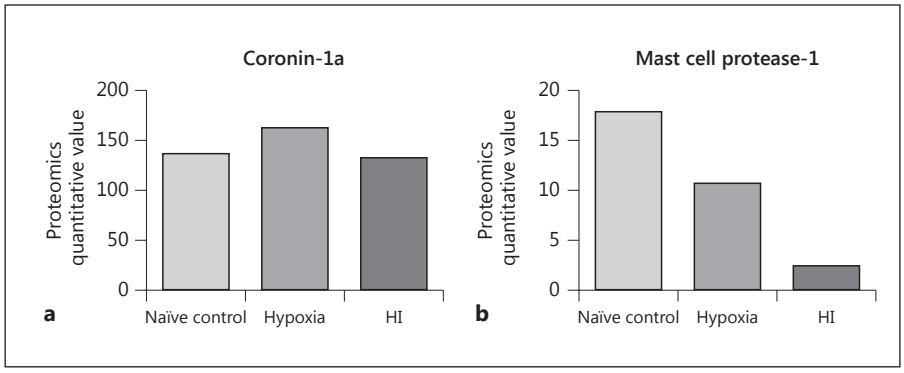


Fig. 5. Proteomic analysis of isolated mast cells: effect of hypoxia and HI on two proteins. **a** Coronin-1a was detected at equivalent levels in all three groups. **b** Mast cell protease-1 was greatest in the mast cells isolated from naïve control rat brain, with significant decreases following HI.

preparation, we did not observe a significant number of cells that were unstained. After the nonadherent mast cells were removed from the culture, there did not appear to be any adherent cells and a thorough trypsin digestion with scraping did not yield any protein (data not shown). However, it was still important to establish the purity of the preparation. The most likely contaminant of this isolation would be microglial cells, which also express Fc receptors, although Fcε is not a major microglial Fc protein [21]. The Western blot of figure 2b demonstrates that Iba-1 was detected at the predicted 17-kDa band in HMC-1 cells and the isolated rat mast cells, as well as in our primary microglial cells, and a more prominent 50-kDa protein in the latter 2 samples. Iba-1 is an actin-binding protein forming a covalent complex [22], and the right-hand panel of figure 2b demonstrates significant actin in all of these preparations, suggesting that this may well be the explanation for the detection of Iba-1 at 50 kDa in the isolated mast cells and microglia.

Iba-1 is routinely considered to be a microglia-specific protein. The results of the Western blot either suggested that mast cells also express this protein or that there may be a significant microglial contamination of the isolated preparation. The latter case would significantly limit the usefulness of the cells obtained via this technique for further study of mast cell function. For final confirmation that mast cells also express Iba-1, we subjected both HMC-1 cells and isolated rat brain mast cells to double immunohistochemistry for avidin, a mast cell granule-specific protein, and Iba-1. Figure 3 demonstrates that both of these mast cell preparations express Iba-1. This result suggests that the positive signal obtained in the Western blot of figure 2 is due to mast cell Iba-1 and not microglial contamination. Iba-1-positive mast cells are also detected in vivo following HI in the P9 rat (fig. 4); however, not all mast cells expressed this protein. Double-labeling of Iba-1 and TB was most often seen in mast cells in the pia and less often in the brain parenchyma.

Preliminary Proteomic Analysis of Isolated Mast Cells: Effect of Hypoxia and HI

Mast cells were isolated from brains of neonatal rat pups at 24 h following unilateral HI, and the samples were subjected to proteomic analysis as described. A complete bioinformatic analysis of these results is well beyond the scope of this initial study. However, figure 5 depicts 2 of the detected proteins of interest. Coronin-1a is an actin-binding protein, which was highly expressed in the isolated mast cells from all of the animals (fig. 5a). Coronin-1a is expressed in hematopoietic cells and, in the brain, has also been attributed solely to microglial cells [23]. Figure 5b depicts the results for the mast cell-specific protein, mast cell protease-1. The mast cells isolated from hypoxic, and hypoxic-ischemic, brain had significantly less of this protease than was detected in the naïve control brain. As more of the cells from the latter 2 isolations showed evidence of degranulation, this might explain the apparent lower value for this soluble enzyme.

Discussion

The primary objective of this study was the development of a methodology to isolate mast cells from neonatal rat brain. Mast cells have previously been isolated from a number of both rodent and human tissues, such as lung and skin, where the in vivo functions of mast cells have been well studied [24], as opposed to brain, where the role of mast cells in several pathological states has been appreciated much more recently. Given the well-established heterogeneity of mast cells, especially in regard to their

responses to different stimuli for migration, activation, and mediator synthesis, it is important to study these functions in mast cells derived from the site of interest, i.e. the brain. To this end, we have adapted a previously described technique based on positive selection with magnetic beads conjugated to an antibody recognizing a major mast cell surface receptor, FcεR1 [17, 25]. With this technique, we are able to isolate TB-positive mast cells from neonatal rat brain in the basal/naïve state, as well as following hypoxia or HI. In addition, we observed that brain mast cells express the protein Iba-1, which is normally used to specifically identify microglia in brain.

FcεR1 is a member of the immunoglobulin receptor superfamily that binds the immunoglobulin IgE, and is highly expressed by mast cells, as well as basophils, human antigen-presenting cells, monocytes, eosinophils and platelets [26, 27]. The FcRs were originally identified as cell surface receptors in immune cells (lymphocytes, macrophages and mast cells) that bind specific immunoglobulins and initiate the classic effector responses of the inflammatory cascade including cytokine production/release and phagocytosis (reviewed in Okun et al. [21]). More recently, expression of one or more of the FcRs has been detected in neural cells, including microglia, neurons, astrocytes, and oligodendrocytes, and the involvement of these receptors in a number of neurologic disorders from stroke to neurodegenerative disease has been reported. However, none of these cells, with the possible exception of microglia, have been reported to express the FcεR. Microglia have been reported to express all FcRs, although the FcγRs are the primary receptors that have been detected and implicated in microglial function (see Okun et al. [21] and references therein). Thus, it was possible that our technique might also select for microglia, which, like mast cells, increase in number and activation following HI in the neonatal rat brain [28, 29].

To investigate microglial contamination of the mast cells, we chose to measure the expression of the protein Iba-1 by Western blot. Iba-1 (ionized calcium-binding adaptor molecule) is a 17-kDa protein that has been described as being specifically expressed in microglia/macrophages and is upregulated upon activation of these cells [30, 31]. The detection of bands at 17 kDa in both the human mast cell line (HMC-1) and in isolated mast cells suggests that Iba-1 is not exclusive to microglia. Interestingly, there was also a strong 50-kDa band in the rat mast cells and microglia. Iba-1 has been shown to be an actin-binding protein involved in the regulation of the reorganization of the actin filament through its actin cross-linking activity [22]. β-Actin was detected as a strong 50-kDa band in all 3 samples (fig. 2b), and although this does not prove that the comparable signal seen for Iba-1 in both isolated rat mast cells and rat microglia is due to actin cross-linking, it could provide an explanation. This line of reasoning would also suggest that the lack of a 50-kDa band for Iba-1 in the HMC-1 cells implies that this aspect of reorganization of the actin cytoskeleton may not be active in this cell line.

Immunofluorescence for mast cells (avidin) and Iba-1 detected positive signals in both the HMC-1 human mast cell line, as well as in isolated rat mast cells (fig. 4), further confirming positive expression of this protein in mast cells. Detection of Iba-1-positive mast cells in situ in tissue sections obtained from neonatal rat brain following HI is more challenging primarily due to the far greater number of microglial cells in these sections. However, several Iba-1-positive/TB-positive cells were detected, primarily in the pial membranes in these sections, as shown in figure 5. Not all TB-positive cells were also positive for Iba-1, suggesting that even within the brain, mast cells are still a heterogeneous population.

We subjected mast cells from naïve control, hypoxic and hypoxic-ischemic neonatal rat brain to broad proteomic analysis and were able to detect a wide variety of proteins differentially expressed under these conditions. Although a complete bioinformatics analysis of these data is well beyond the scope of this paper, we present 2 examples of such detection (fig. 5). Of note, coronin-1a, which is an actin-regulatory protein, is very highly expressed in all of the samples. This is in good agreement with previous studies demonstrating involvement of both coronin-1a and coronin-1b in rearrangement of the mast cell cytoskeleton during degranulation and cytokine secretion [32]. In the brain, coronin-1a has been described as a microglia marker, similar to Iba-1 [23]. The results reported here suggest that the use of both markers should be interpreted carefully in any situation in which the mast cell population is increased/activated in the brain.

In conclusion, this study establishes a methodology for the successful isolation of mast cells from the immature rodent brain under control, as well as following hypoxic and hypoxic-ischemic exposure. The isolated mast cells provide a useful substrate for specific protein analysis of mast cell synthetic activity in vivo. In addition, these cells will be useful for further in vitro studies of cell-to-cell interaction to further study mast cell interaction with neural cells under a wide array of physiologic and pathophysiologic conditions.

Acknowledgments

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