HMG-2010-D-00986, Ambrosini

SUPPLEMENTARY MATERIAL AND METHODS

Immunofluorescence

Astrocytes were incubated over night at 4°C with anti-MLC1 pAb (1:50, Atlas AB) and anti-Rab5 (D-11) mAb (1:25, Santa Cruz Biotecnology) and then for 1 h at RT with a rhodamine-conjugated donkey anti-goat (1:100, Jackson, UK) and a fluorescein-conjugated donkey anti-mouse (1:100, Jackson, UK) antibody. For double staining with anti-EEA1 mAb (1:50, BD Transduction Laboratories) and anti-Kir4.1 pAb (1:50, Alomone), cells were incubated for 1 h at RT. A biotinilated secondary antibody (4.3 μg/ml ,Biotin-SP-AffiniPure goat anti-rabbit IgG H+L, Jackson Immunoresearch Laboratories) followed by incubation with 2 μg/ml streptavidin-TRITC (Jackson, UK) and a fluorescein-conjugated donkey anti-mouse (1:100, Jackson, UK) were used. Coverslips were washed, sealed in Vectashield medium (Vector Lab, Burlingame, CA) and analyzed with a laser scanning confocal microscope as previously described in Materials and Methods.

Immunohistochemistry of human brain tissue

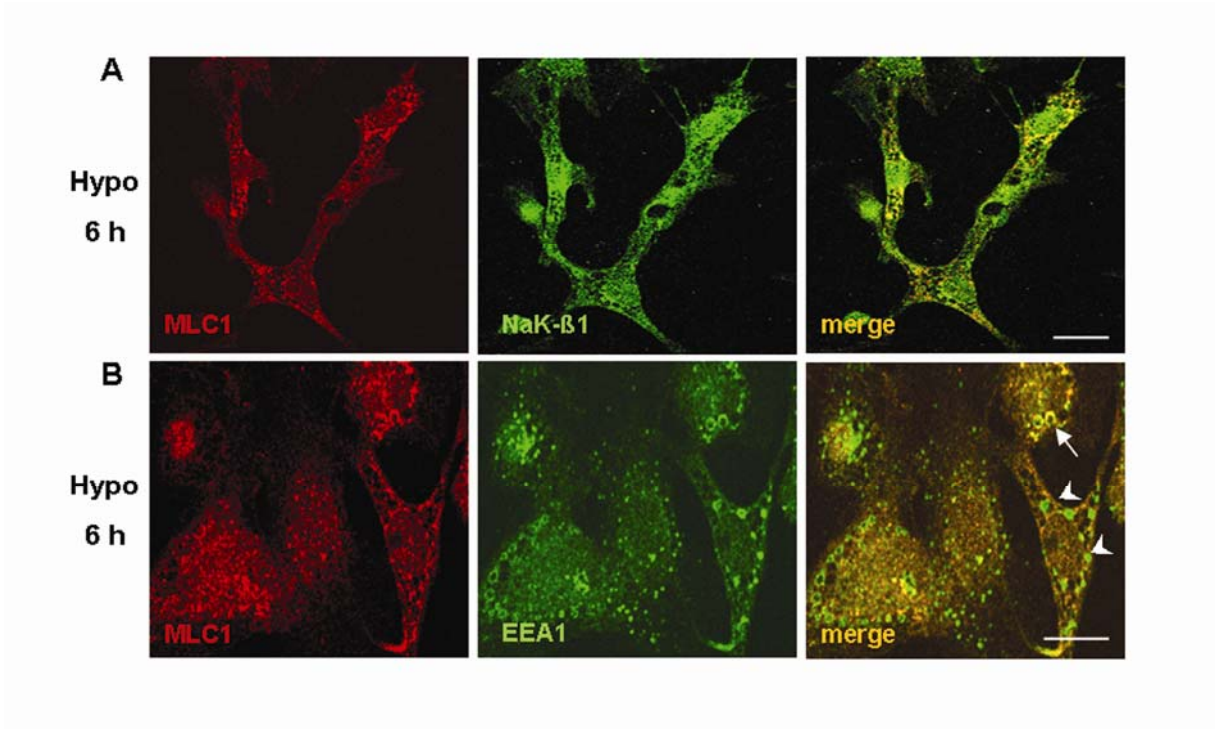
Indirect immunofluorescence technique was used to detect MLC1 in post-mortem brain tissues from a control patient without neurological disease and from a patient with multiple sclerosis (MS) obtained from the UK MS Tissue Bank at Imperial College London. As described in Materials and Methods, sections were incubated overnight at 4°C with a mixture of rabbit anti-MLC1 pAb (1:250, ATLAS) and anti-Na,K-ATPase β1 mAb (1:200, Millipore). After extensive washing, sections were incubated for 1 h at RT with a mixture of fluorescein-conjugated goat anti-mouse and rhodamine-conjugated goat anti-rabbit Abs and images were analysed with a fluorescent microscope (Leica DM-4000B Microsystems, Bannockburn, IL).

Supplementary figures

Figure S1. Characterization of vacuoles induced by 6h hypo-osmotic treatment of rat astrocytes. (A) Double immunofluorescence stainings with anti-MLC1 pAb (red) and anti-Na,K-β1 mAb (green) show partial colocalization of MLC1 and NaK-β1 in the membranes bordering the newly formed vacuoles. (B) Double immunostainings with anti-MLC1 pAb (red) and anti-EEA1 mAb (green), a marker of early endosomes, reveal a partial colocalization of MLC1 and EEA1 around some vacuoles (Hypo 6h, arrow) while others are immunopositive only for EEA1 (Hypo 6h, arrowhead). Scale bars: 20 μm

Figure S2. Characterization of vacuoles induced by twelve hours hypo-osmotic treatment of rat astrocytes. (A) In astrocytes treated for 12 h with hypo-osmotic medium there is a high level of colocalization between MLC1 (red) and Rab5 (green), a marker of the early endosomes, along the vacuole limiting membranes and in the perinuclear area. (B) Staining with anti-Kir4.1 pAb (red) and anti-EEA1 mAb (green) shows Kir4.1 and EEA1 colocalization along vacuole membranes. Scale bars: 20 μm

Figure S3. Immunohistochemical detection of MLC1 in non-pathological human brain (A) and in multiple sclerosis (MS)-affected brain (B). (A) Immunofluorescence staining of a control human brain section with anti-MLC1 pAb shows strong immunoreactivity around blood vessels (asterisks) in the periventricular white matter. (B) In the MS brain MLC1 is more abundantly expressed in perivascular areas of an active white matter lesion. Numerous small blood vessels appeared positive for MLC1. Scale bars: 200 μm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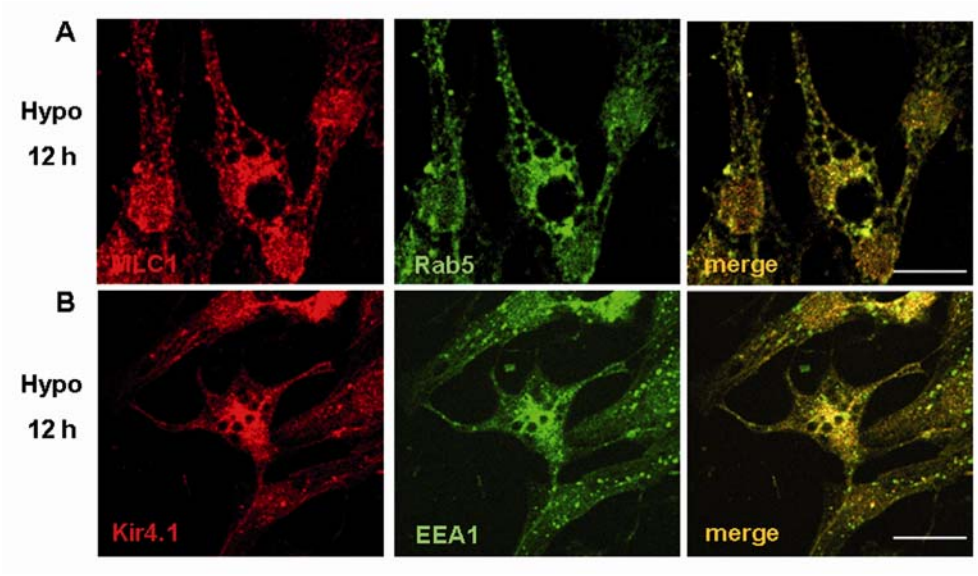
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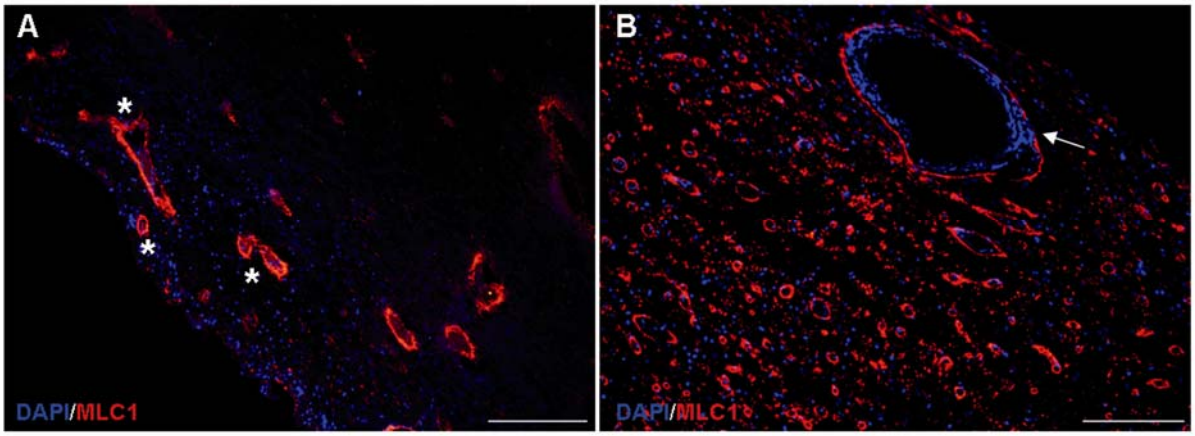
A
B
DAPI/MLC1
DAPI/MLC1