C38 is a Neuron Specific Mitochondrial Protein that Controls Neuronal Development

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Abstract:
The retina has a well-organized cellular structure and belongs to the central nervous system. The retina has been used as a model of the central nervous system, especially neuronal degeneration and axonal regeneration. Monoclonal C38 antibody has been used as a molecular marker of retinal ganglion cells, but the molecular function of its antigen has not been clarified. A recent study has demonstrated that C38 is a neuron-specific mitochondrial protein that enhances neuronal maturation. This review outlines the functions of C38, as investigated through a retina model.

Keywords: C38, Commitment, Differentiation, Horizonal cells, Maturation, Retinal ganglion cells

1. Introduction

During ontogeny, the retina derives from the developing brain and belongs to the central nervous system (CNS). Because of its highly organized cellular architecture and relative simplicity, the retina has been used as a representative model for studying neuronal cells of the CNS. There are additional advantages in studying the retina as a model of the CNS, especially for studying neuronal development, cell death and regeneration2). In a previous study, we have isolated a neuron-specific molecule from the retina and showed that it is specifically localized in neurons not only in the
retina but also in the brain and spinal cord\textsuperscript{2}. By using the retina as a model of the CNS, we can clarify its molecular function more precisely. Here, the functions of this molecule in the retina are described, as well as the advantages in using the retina as a model of the CNS.

2. C38 labels retinal ganglion cells and enhances neuronal maturation

2-1. Anatomical structure of the retina

The neural retina is approximately 0.5 mm thick. A vertical section of the retina reveals that there are three nuclear layers (Fig. 1). The photoreceptors lie outermost in the retina against the pigment epithelium and choroid and their nuclei form the outer nuclear layer (ONL). The inner nuclear layer (INL) consists of the nuclei of bipolar cells and interneurons, including amacrine cells and horizontal cells. The innermost nuclear layer of the retina, facing the vitreous and closest to the lens, consists mostly of retinal ganglion cells (RGCs) and is the ganglion cell layer (GCL). The synapses between each nuclear layer form the inner and outer plexiform layers. Light stimulus received by the photoreceptors is transduced as an electrical signal and transmitted to the RGCs via bipolar cells. The major retinal glia are Müller cells and their nuclei localize in the INL. The inner limiting membrane (ILM) is formed by Müller cell end feet and the outer limiting membrane (OLM) is formed from adherens junctions between inner segments of photoreceptors and Müller cells.

2-2. The developmental generation of retinal neurons

During retinal development, retinal neurons are generated sequentially from multipotent retinal progenitor cells similar to other CNS neurons. Horizontal cells and RGCs are the first populations generated from the retinal progenitor cells and they become post-mitotic at embryonic day 16 (E16) and E18 in rat and mouse, respectively\textsuperscript{3,4}. Amacrine cells and photoreceptor cells are generated in a broad window of the retinal development, from the middle of the embryonic stage to the postnatal period. Bipolar cells and Müller cells are the last populations generated and develop predominantly in the postnatal period, until around postnatal 1 week. Retinogenesis completes until P14.

2-3. C38, a neuron-specific molecule specifically expressed in RGCs in the retina

Cell death, survival and axonal regeneration of RGCs after optic transection have been widely investigated in many laboratories as a representative model of CNS neurons because of their anatomical feature\textsuperscript{5}. As RGCs extend their axons into the optic nerve and their somata are present in the retina, on optic nerve transection, only the axons of RGCs can be cut without damage to the somata of RGCs. In contrast, in spinal cord or brain, when the axons are transected, their somata are also damaged. Thus, the retina is an ideal model for these experiments. However, in the GCL, there are a large number of displaced amacrine cells, which are present in addition to RGCs. As almost half of the neurons are displaced amacrine cells\textsuperscript{6}, identification of RGCs is especially important. However, only a few RGC-specific markers are known. We have developed monoclonal antibody C38 using rat retina as an immunogen by immunological subtraction method\textsuperscript{2}. C38 antigen molecule is a 24 kDa protein consisting from 149 amino acids in rat (accession #NP_001014185). The amino acid sequences of mouse and human C38 are 97 and 79% identical to the rat C38, respectively. Monoclonal antibody C38 is an RGC-specific marker that can label most, if not all, RGCs in cat\textsuperscript{2}, ferret\textsuperscript{7}, rat\textsuperscript{2}, mouse, hamster and rabbit (unpublished data). In cat and ferret, this antibody specifically labels RGCs; thus, we can discriminate RGCs from displaced amacrine cells (Fig. 2a)\textsuperscript{2,7}. In addition, using this antibody, we can easily recognize the subtypes of RGCs (Fig. 2b). In the rat retina, C38 antibody labels horizontal cells as well as RGCs...
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During retinogenesis of rat, RGCs and horizontal cells are generated during the prenatal period at relatively similar developmental stages. However, the expression patterns of C38 in these cells show significant differences (Fig. 3). C38 is first expressed in RGCs from E16, as faint staining is observed in the GCL and the nerve fiber layer (NFL). During the prenatal period, no immunoreactivity of C38 is detected in the neuroblastic layer (NBL), where horizontal cells are present at this stage. The immunoreactivity of horizontal cells in the NBL is first detected from P0 (Fig. 3). C38 immunoreactivities increase during postnatal development and until P14, when almost similar staining patterns as in adults is observed.

Both RGCs and horizontal cells become post-mitotic by E18 at the latest\textsuperscript{3,4}. The expression pattern of C38 in RGCs and horizontal cells indicates that C38 is expressed in the post-mitotic neurons\textsuperscript{10}. Then, why is C38 not expressed in prenatal post-mitotic horizontal cells? One possible explanation is that C38 expression correlates with neuronal maturation, rather than terminal mitosis and cell fate determination. RGCs begin neuronal maturation immediately after their final mitosis. In contrast, post-mitotic horizontal cells remain in a relatively immature state during the prenatal period and the beginning of maturation is protracted until the postnatal period\textsuperscript{11}. Accordingly, final mitosis is completed at a relatively similar age in both horizontal cells and RGCs, but maturation of RGCs begins significantly earlier than that of horizontal cells\textsuperscript{11,12}. As C38 expression correlates with neuronal maturation, rather than terminal mitosis and cell fate determination, C38 is not expressed in immature prenatal horizontal cells but begins to be expressed in relatively mature postnatal horizontal cells. This is supported by in vitro studies using P19 embryonal carcinoma (P19EC) cells, which can differentiate into neuronal cells by stimulation with appropriate concentrations of retinoic acid. C38 over-expression alone cannot reduce cell proliferation nor induce neuronal fate determination of P19EC cells. However, when cell fate is committed to neurons by retinoic acid stimulation, C38 enhances neuronal maturation of P19EC cells\textsuperscript{10}.

BM88, which was isolated from porcine brain and is identical to C38, is reported to down-regulate proliferation and enhance neuronal differentiation of neural progenitor cells derived from embryonic spinal cord as well as adult neural precursors in the subventricular zone\textsuperscript{13,14}. However, in these cells, cell fate determination and the following neuronal maturation are a continuing phenomenon and it is difficult to distinguish between them. Accordingly, it may be difficult to investigate the functions of a molecule in these cells to determine whether the molecule reduces cell proliferation, promotes fate determination and enhances neuronal maturation or has just one of these functions. In contrast, in the retina, we can observe both RGC and horizontal cells in

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**Fig. 2.** Localization of C38 antigen labeled by C38 antibody. Immunohistochemical study of retinas. (a) Vertical section of ferret retina. C38 localizes only in the NFL, the GCL and the INL. Bar = 50 μm. (b) Flatmount preparation and the three-dimensional section of ferret retina. C38 antibody specifically labels RGCs. In addition, the morphological characteristics of both α- and β-subtype RGCs are readily identified. Bar = 10 μm. (c) Vertical section of rat retina. C38 antibody labels the OPL in addition to the NFL, the GCL and the IPL. Bar = 50 μm.
Fig. 3. Developmental expression of C38 during retinal development of rat.
C38 is first detected in the GCL from E16. From P0, C38 immunoreactivity is also observed in the NBL with fine neurites extending to vitreous (arrows) and ventricular (arrowheads) side. C38 positive cells in the NBL extend horizontal processes obviously from P5 and divide the NBL into the INL and the OPL. Until P14, the C38 expression patterns are almost similar to the adult retina. Bar = 50 μm.

Fig. 4. C38 enhances neuronal maturation of P19 embryonal carcinoma (P19EC) cells in the presence of retinoic acid. P19EC cells can differentiate into neuron-like cells by stimulation from high concentrations (100 nM) of retinoic acid (RA). Immunohistochemistry using doublecortin (DCX) (a, b) or microtubule-associated protein 2 (MAP2) antibodies. (a) When stimulated by low concentrations of (5 nM) RA, wild type P19EC cells cannot express the immature neuron-specific marker DCX\(^{11}\). (b) Without RA stimulation, P19EC cells over-expressing C38 solely cannot differentiate into neurons. (c) In combination with C38 and low concentration of RA, P19EC cells differentiate into mature neurons and express MAP2. Bar = 50 μm.
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the same specimen and can compare the functional differences of the molecules between them. This will be advantageous when using the retina as a representative model of neuronal tissues, especially in neuronal development.

3. Perspectives of C38

At the subcellular level, C38 is localized in mitochondria (Fig. 5). As C38 is a neuron-specific molecule, C38 is one of the neuron-specific mitochondrial proteins. However, there have been only a few neuron-specific mitochondrial proteins reported to date (Mitochondrial Proteome Database for mitochondria-related genes, proteins and disease; URL: http://www.mitop.de:8080/mitop2/). Mitochondria are known to have crucial roles in controlling cell survival and death. More recently, it has been shown that mitochondria are not stationary organelles\(^{15}\). Instead, they change their morphology dynamically through the balance in fusion and fission (division), and these processes are strictly controlled in response to cellular conditions including energy demand and cell cycle. Mitochondrial dynamics are also essential for appropriate embryonic development, including nervous system development\(^{16,17}\). It is still unknown how C38 enhances neuronal maturation; however, as it selectively localizes in the mitochondria, C38 might exhibit its functional properties through mitochondria. In addition, as C38 is a neuron-specific mitochondrial protein and enhances neuronal maturation, elucidation of this mechanism may result in new insight into mitochondrial functions.

C38 expression levels are different between RGC subtypes. C38 is highly expressed in RGCs with larger cell soma when compared to the cells with smaller soma (Fig. 6). There are several subtypes of RGCs and the predominant types are \(\alpha\) subtypes, with larger soma and widespread dendrites, and \(\beta\) subtypes, with smaller soma and narrow dendrites, as shown in Figure 2b. After optic nerve transection, \(\alpha\) type RGCs have a higher capacity for survival than \(\beta\) type cells\(^{18}\). In autosomal dominant optic atrophy, which is caused by mutation in mitochondrial protein OPA1, \(\beta\) type RGCs are also affected predominantly\(^{19}\). In contrast, in glaucoma, larger cells die first as compared to smaller cells\(^{20}\). However, the reasons for subtype-specific vulnerability in specific pathological conditions are still not known. Because of the subtype-specific distribution of C38, the reason(s) for subtype-specific cell death may be clarified by investigating C38 functions in the future.

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Fig. 5. Subcellular localization of C38.

C38 expression vector was transfected into Chinese Hamster Ovary cells. C38 and GRP75, a mitochondria-specific marker, are co-immunolabeled. C38 and GRP75 are colocalized at subcellular level. Nucleus is stained with Hoechst33258. Bar = 10 \(\mu\)m.

Fig. 6. C38 is highly expressed in larger RGCs.

The difference of C38 immunoreactivity among the RGCs. Vertical section of ferret retina was immunolabeled with C38 antibody. Only the GCL is shown. As indicated by arrows, RGCs with larger somata are strongly labeled by C38 antibody when compared to the smaller RGCs (arrowheads). Nuclei are stained with Hoechst33258. Bar = 10 \(\mu\)m.
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