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Temporal regulation of CD81 following retinal injury in the rat

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Abstract

Following retinal injury, glial cells within the retina undergo a response that is characterized by the proliferation of astrocytes, Müller cells, and retinal pigment epithelial cells. CD81, a small membrane protein known to be involved in cell proliferation, is up-regulated after injury. This study focuses on the temporal regulation of CD81, relating the expression of this protein to glial fibrillary acidic protein (GFAP), the classic marker of gliosis. CD81 levels were elevated at 7 days after injury and remained elevated at 30 days after injury; GFAP was increased at 7 days and continued to increase until 30 days post injury. This association of CD81 with glial reactivity may provide a clue to the regulation of the proliferative response following retinal injury.

Keywords

CD81; Glial fibrillary acidic protein; Retina; Injury; Tetraspanin; Gliosis; Proliferation

The response of the retina to trauma involves well-characterized cellular reactions [16], many of them similar to responses that occur in other parts of the central nervous system. As in the brain and spinal cord, a dramatic up-regulation of glial fibrillary acidic protein (GFAP) follows retinal injury. This up-regulation of GFAP marks reactive retinal astrocytes [12,16], which hypertrophy and increase their expression of the intermediate filament protein GFAP [3,13, 18]. Müller glia and the retinal pigment epithelium (RPE), which are unique to the retina, also demonstrate characteristic responses to injury. Müller cells, like astrocytes, up-regulate GFAP. In addition, both Müller glia cells and RPE cells can proliferate to form scar tissues within the retina [14]. They also can migrate into the vitreal space, proliferate, and form cellular membranes [7], a response known as proliferative vitreoretinopathy [11]. These cellular membranes can then contract and cause secondary retinal detachments. The reactive glial responses and the proliferation of non-neuronal cells can have serious consequences, including loss of sight.

Studies in our laboratory have identified a protein, CD81, which plays a role in the response of the brain [9], spinal cord [5], and retina [10]. We originally called CD81 the target of the antiproliferative antibody. As that name implies, this protein is part of a molecular complex that controls the proliferation of glial cells. We have hypothesized that this CD81 molecular complex is a key element in the contact inhibition between glial cells in the brain. Antibodies directed against CD81 depress the mitotic activity of cultured cells [9,17]. Furthermore, CD81 is expressed by glial cells in the brain during the second postnatal week, a time when their mitotic activity is down-regulated [19]. The antiproliferative role of this protein is confirmed by mice with a CD81-null mutation [10]. These CD81-null mice have very large brains containing an over abundance of astrocytes and microglia [10]. We have found that CD81 is

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dramatically up-regulated following injury to the brain, spinal cord, and retina. Furthermore, there is a tight correlation between the up-regulation of CD81 and reactive gliosis. The present study focuses on the temporal regulation of CD81 following retinal injury, comparing the level of this protein to that of GFAP and other known markers of injury in the retina.

In this study, we used the monoclonal antibody AMP1 to recognize CD81 [9]. A polyclonal antibody purchased from Lipshaw (Catalog Number 460740) and a monoclonal antibody TED1 [8] were used to identify GFAP. A monoclonal mouse anti-cytokeratin, AE1/AE3 (MAB 3412), and anti-cytokeratin (CBL 234) from Chemicon were used to recognize cytokeratin. The peroxidase-conjugated secondary antibodies were goat anti-mouse IgG and goat anti-rabbit IgG. All secondary antibodies, purchased from Jackson ImmunoResearch Laboratories, were absorbed against serum proteins and were specific to the IgG of the species used as a primary antibody.

We used 17 adult Sprague–Dawley (albino) rats, eight for immunohistochemistry and nine for immunoblot analysis. Rats were anesthetized with a mixture of xyaline (13 mg/kg), and ketamine (87 mg/kg). We then made bilateral tears in the retinas of each of ten rats by inserting a 22-gauge needle through the temporal pars plana and scraping the nasal retina. The animals were allowed to recover from anesthesia and then were returned to the animal care facility. The Animal Care and Use Committee of the University of Tennessee Health Science Center approved all animal protocols used in this study.

At 1, 3, 7, and 30 days after the placement of retinal tears, rats were deeply anesthetized with xyaline (26 mg/kg) and ketamine (174 mg/kg). They were then perfused through the heart with 0.1 M phosphate-buffered saline (pH 7.5), followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The intact globes were excised and placed in 30% sucrose in phosphate buffer. The eyes were sectioned on a cryostat at a thickness of 15 μ m. The details of the subsequent immunohistochemical procedures are described in Geisert et al. [10]. In brief, the sections were blocked for 1 h in borate buffered saline containing 4% BSA with 0.1% DMSO. They were then incubated overnight in primary antibody at 4 °C, then rinsed and placed in the secondary antibody for 2 h at room temperature. After three rinses, the sections were reacted with diaminobenzidine and hydrogen peroxide.

In a previous study [4], we found that CD81 was dramatically increased at 14 days after injury. The bodies of cells in the ganglion cell layer were outlined, as was the inner plexiform layer, while the cell bodies in the inner nuclear layer were even more distinctly outlined. When sections were stained for GFAP, a dramatic pattern of vertical processes was observed. This pattern is characteristic of reactive Müller cells within the injured retina. We extended these observations by examining the levels and distribution of markers of retinal astrocytes, Müller cells and RPE at several different time points after an experimentally induced retinal tear.

In the present study, immunohistochemical staining of the retina revealed a modest increase in CD81 immunoreactivity on the first day after injury. Most of this increase was observed at the site of injury. At three days after injury, the level of CD81 had increased. At 7 days, there was a distinct elevation of CD81 immunoreactivity at the cellular scar (Fig. 1B,D) and throughout the retina. At 30 days, elevated levels of CD81 were observed at the region of injury with a distinct concentration of CD81 reaction product next to the vitreal surface.

The levels and distribution of CD81 were compared to those of GFAP and cytokeratin. At one day after injury, there was a distinct increase in GFAP staining in the immediate vicinity of the injury. Astrocytes in the ganglion cell layer showed GFAP immunoreactivity, while Müller cells at the site of injury expressed modest levels of GFAP. Away from retinal tears, the Müller cells were not noticeably GFAP-positive. The levels of GFAP immunoreactivity increased with time. From the third day after injury, both astrocytes and Müller cells showed up-regulation

of GFAP similar to that previously described [12,15,16]. The levels of GFAP continued to increase so that, by 7 days after injury, two distinct cellular responses were observed at the site of injury. The first response consisted of cells that were GFAP-positive (Fig. 1A). These cells appeared to be reactive Müller cells. In adjacent sections stained for CD81, elevated levels of immunoreactivity were present at the site of injury (Fig. 1B). This elevation of reaction product was not observed in control sections (data not shown). At other injury sites a second type of response was observed. The cells forming the scar and filling the tear are GFAP-negative (Fig. 1C). These cells may be retinal pigment epithelial cells, which in albino rats are difficult to identify. However, in sections stained for cytokeratin a modest increase in immunoreactivity was observed in the GFAP-negative cells (data not shown). These GFAP-negative regions, like the GFAP-positive regions, are high in CD81 immunoreactivity (Fig. 1D).

Based on the immunohistochemical analysis, it appeared that the levels of GFAP and CD81 had increased in response to retinal injury. To estimate the extent of protein up-regulation, we examined the levels of CD81, GFAP, and cytokeratin using a quantitative immunoblot analysis (Fig. 2). Protein samples were taken from the normal retina. These samples were compared to samples taken 7 and 30 days after experimentally induced tears. For each sample, both retinas from a single animal were pooled and placed in non-reducing sample buffer (2% SDS, 10% glycerol in 0.05 M Tris-HCl buffer, pH 6.8). Three rats were used at each time point. A quantitative immunoblot method was similar to that previously described [9]. The protein samples were balanced and equal loads of proteins were run on 5% to 16% acrylamide gels. The proteins were then transferred to nitrocellulose. The blots were blocked in borate buffer (pH 8.4) containing 5% nonfat dry milk and probed with the primary antibodies overnight at 4 °C. After rinsing in borate buffer, the blots were incubated in peroxidase-labeled secondary antibody (1:500) for 2 h at room temperature or overnight at 4 °C, rinsed extensively, and reacted with 0.05% 3,3' diaminobenzidine tetrahydrochloride (Sigma) in 0.05 M Tris buffer (pH 7.3) and 0.01% hydrogen peroxide. The level of protein expression was defined by quantifying the intensity of the immunopositive bands on the blots. Blots were scanned using a Macintosh computer with a high resolution desktop scanner (Hewlett Packard ScanJet 5370C). NIH Image software was then used to compare the intensity of the immunolabeling. The level of each protein band was defined in relative absorbance units (AU).

As shown in Fig. 2, there was a 1.7-fold increase in CD81 at 7 days after injury (52.94 ± 6.64 AU, mean $\pm$ SD). This amount of CD81 was significantly greater (Mann-Whitney *U*-test, $P < 0.05$) than that in the normal retina (31.97 ± 8.87 AU). The increase in CD81 observed at 7 days was maintained up to 30 days after injury (44.2 ± 17.4 AU). The difference between these two time points was not significant. The 1.5-fold increase (119.6 ± 1.8 AU) in GFAP at 7 days after injury attained significance ($P < 0.01$). The levels of GFAP continued to increase up to 30 days after injury (151.93 ± 9.17 AU, $P < 0.01$), with the normal value being 77.3 ± 15.2 AU. When data from all three cytokeratin bands (at 48 kDa, 50 kDa, and 56.5 kDa) were combined, there was an approximately 2.5-fold increase (31.17 ± 11.50 AU, $P < 0.05$) at 7 days after injury and a 3-fold increase (38.81 ± 3.53 AU) at 30 days after injury, while the level in the normal retina was 11.64 ± 6.11 AU.

These data support our immunochemical finding of an up-regulation of CD81, GFAP, and cytokeratin in the retina after injury. In fact, because the protein samples were obtained from whole retina, of which the injury site constituted only a small portion, this determination of protein levels underestimates the actual up-regulation of these proteins at the site of injury. Thus, the up-regulation of CD81, GFAP, and cytokeratin at retinal tears was considerably greater than our immunoblot data indicate.

The response of the retina to injury involves different non-neuronal cell types. Astrocytes and Müller cells become reactive, expressing high levels of GFAP. In this study, as in previous

ones [4], we found elevated levels of GFAP on the first day after placement of retinal tears. Moreover, the levels of GFAP continued to increase over time, labeling astrocytes and Müller cells. This was confirmed by our immunoblot analysis, which demonstrated not only a dramatic elevation of GFAP at 7 days after injury but also a continued increase up to 30 days after injury. RPE can dedifferentiate, proliferate, and migrate into the retina and vitreal space, forming cellular membranes [11,12]. We noted many occurrences of the formation of cellular membranes at the site of injury and the extension of these membranes into the vitreous. Resting microglia can become activated and, along with invading peripheral blood components, mount an inflammatory response [6]. All of these non-neuronal cells that are associated with the response of the retina to injury are known to express CD81 [5,6,9,17]. Thus, CD81 may play a critical role in different cellular responses after injury.

In previous studies, we have shown that CD81 associates with reactive gliosis in the brain [9], spinal cord [5], and retina [10]. The present study demonstrates that CD81 is temporally regulated after retinal injury. We believe that CD81 is an integral part of the reactive response of the retina to injury, being up-regulated by astrocytes, Müller cells, and RPE [4,10]. CD81 belongs to a family of proteins, the tetraspanins, which are known to form molecular complexes within the plane of the membrane. These tetraspanin complexes link extracellular interactions to the second messenger system. For example, CD81 and other tetraspanins can form a molecular complex with integrins to regulate interactions of the cell with the extracellular matrix, affecting second messenger systems (phosphatidylinositol 4-kinase) and eventually modulating the mitotic activity of the cell [1,2,20]. To date, the data indicate that CD81 is part of a molecular switch that controls reactive gliosis and the proliferation of non-neuronal elements in the retina.

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References

1. Berditchevski F, Tolias KF, Wong K, Carpenter CL, Hemler ME. A novel link between integrins, transmembrane-4 superfamily proteins (CD63 and CD81), and phosphatidylinositol 4-kinase. *J Biol Chem* 1997;272:2595–2598. [PubMed: 9006891]
2. Boucheix C, Rubinstein E. Tetraspanins. *Cell Mol Life Sci* 2001;58:1189–1205. [PubMed: 11577978]
3. Bignami A, Dahl D. The radial glia of Müller in the rat retina and their response to injury: an immunofluorescence study with antibodies to the glial fibrillary acidic (GFA) protein. *Exp Eye Res* 1979;28:63–69. [PubMed: 376324]
4. Clarke K, Geisert EE Jr. The target of the antiproliferative antibody (TAPA) in the normal and injured rat retina. *Mol Vis* 1998;4:3. [PubMed: 9485486]
5. Dijkstra S, Geisert EE Jr, Gispén WH, Bar PR, Joosten EAJ. Up-regulation of CD81 (target of the antiproliferative antibody; TAPA) by reactive microglia and astrocytes after spinal cord injury in the rat. *J Comp Neurol* 2000;428:266–277. [PubMed: 11064366]
6. Dijkstra S, Geisert EE Jr, Dijkstra CD, Bar PR, Joosten EAJ. CD81 and microglial activation in vitro: proliferation, phagocytosis and nitric oxide production. *J Neuroimmunol* 2001;114:151–159. [PubMed: 11240026]
7. Fisher SK, Erickson PA, Lewis GP, Anderson DH. Intraretinal proliferation induced by retinal detachment. *Invest Ophthalmol Vis Sci* 1991;32:1739–1748. [PubMed: 2032796]
8. Geisert EE Jr, Stewart AM. Changing interactions between astrocytes and neurons during CNS maturation. *Dev Biol* 1991;143:335–345. [PubMed: 1991556]
9. Geisert EE Jr, Yang L, Irwin MH. Astrocyte growth, reactivity, and the target of the antiproliferative antibody, TAPA. *J Neurosci* 1996;16:5478–5487. [PubMed: 8757260]

10. Geisert EE Jr, Abel HJ, Fan L, Geisert GR. Retinal pigment epithelium of the rat express CD81, the target of the anti-proliferative antibody (TAPA). *Invest Ophthalmol Vis Sci* 2002;43:274–280. [PubMed: 11773042]
11. Glaser BM, Cardin A, Biscoe B. Proliferative vitreoretinopathy, the mechanism of development of vitreoretinal traction. *Ophthalmology* 1987;94:327–332. [PubMed: 3587912]
12. Guidry C. Tractional force generation by porcine Müller cells. *Invest Ophthalmol Vis Sci* 1997;39:456–468. [PubMed: 9040479]
13. Humphrey MF, Chu Y, Mann K, Rakoczy P. Retinal GFAP and bFGF expression after multiple argon laser photocoagulation injuries assessed by both immunoreactivity and mRNA levels. *Exp Eye Res* 1997;64:361–369. [PubMed: 9196387]
14. Lewis GP, Erickson PA, Guérin CJ, Anderson DH, Fisher SK. Changes in the expression of specific Müller cell proteins during long-term retinal detachment. *Exp Eye Res* 1989;49:93–111. [PubMed: 2503391]
15. Maclaren RE. Development and role of retinal glia in regeneration of ganglion cells following retinal injury. *Br J Ophthalmol* 1996;80:458–464. [PubMed: 8695569]
16. Miller NM, Oberdorfer M. Neuronal and neuroglial responses following retinal lesions in the neonatal rats. *J Comp Neurol* 1981;202:493–501. [PubMed: 7298911]
17. Oren R, Takahashi S, Doss C, Levy R, Levy S. TAPA-I, the target of an antiproliferative antibody, defines a new family of transmembrane proteins. *Mol Cell Biol* 1990;10:4007–4015. [PubMed: 1695320]
18. Sarthy PV, Fu M, Huang J. Developmental expression of the glial fibrillary acidic protein (GFAP) gene in the mouse retina. *Cell Mol Neurobiol* 1991;11:623–637. [PubMed: 1723659]
19. Sullivan CD, Geisert EE Jr. Expression of rat target of the antiproliferative antibody (TAPA) in the developing brain. *J Comp Neurol* 1998;396:366–380. [PubMed: 9624590]
20. Zhang XA, Bontrager AL, Hemler ME. Transmembrane-4 superfamily proteins associate with activated protein kinase C (PKC) and link PKC to specific beta1 integrins. *J Biol Chem* 2001;276:25005–25013. [PubMed: 11325968]

Fig. 1.

The response of the retina to injury is illustrated in four photomicrographs taken 7 days after placement of a retinal tear. Two basic responses to injury can be seen. (A) The site of the induced tear (arrow) is filled with GFAP-positive cells (Müller glia or astrocytes). (B) In an adjacent section, the glial scar demonstrates a substantial increase in CD81 immunostaining. At other sites of the retinal tear, the cells filling the site of injury are GFAP-negative (C) but nonetheless show high levels of CD81 immunoreactivity (D). Note the cellular tissues (white arrows) and erythrocytes (white arrow heads) that extend out into the vitreal space. All of the photomicrographs at the same magnification; the scale bar in D = 100 μ m.

Fig. 2.

The levels of CD81, GFAP and cytokeratin were quantified using immunoblot methods at 7 and 30 days following injury. Note that the levels of all three proteins, expressed in relative absorbance units, increased after injury. CD81 expression was significantly up-regulated at 7 days after injury and remained elevated at 30 days. The levels of GFAP were up-regulated approximately 1.5- and 2-fold at 7 days and 30 days, respectively. Cytokeratin was increased 2.5-fold at 7 days after injury and 3-fold at 30 days after injury. Asterisks denote significant differences from the normal: (Mann-Whitney *U* test, * $P < 0.05$, ** $P < 0.01$).