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## Co-expression of glial fibrillary acidic protein and vimentin in reactive astrocytes following brain injury in rats

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The immunohistochemical expression of glial fibrillary acidic protein (GFAP) and vimentin (VIM) was studied in reactive astrocytes of the rat cerebral cortex 5 days after a brain injury. Seriated Epon semithin sections were immunostained alternatively for GFAP or VIM. Thereafter, both antigens were detected in consecutive sections of the same cell. Bordering the wound, an inner reactive glial layer 300–350  $\mu\text{m}$  thick, showed positive astrocytes with the two immunohistochemical techniques. In this layer, about 60% of the GFAP positive astrocytes were also positive for VIM. Outside the inner layer, only GFAP positive astrocytes could be found.

Differentiating astrocytes show changes in the expression of intermediate filament proteins. Thus, the radial glia and immature astrocytes are positive for vimentin (VIM) and lack of glial fibrillary acidic protein (GFAP)<sup>2,3,6,16,21</sup>. In the course of the first two postnatal weeks a transition from VIM to GFAP takes place<sup>13,16,21</sup>, whereby astrocytes in adult animals are usually positive for GFAP and negative for VIM.

During the transitional period, both VIM and GFAP can be co-expressed. This co-expression is a transient event for most astrocytes in the central nervous system (CNS)<sup>13,15,21</sup>. Nevertheless, yet in the normal adult, astrocytes in specific locations including cerebellum, retina, large tracts of myelinated fibers and optic nerve, still co-express VIM and GFAP<sup>4,13,15,17</sup>.

Gliosis is the most common response to different injuries of the CNS. Numerous studies have demonstrated an enhanced expression of GFAP in reactive astrocytes in lesions of different types<sup>1,2,8,10,11,15,18–20</sup>. Reactive astrocytes appear to recover the capacity to express VIM, lost during normal development<sup>13,14,19</sup>. The co-expression of GFAP and VIM has been described in reactive astrocytes in different experimental models (brain wound in neonatal rats<sup>13</sup>, laser-induced brain necrosis and brain tumors induced by ethylnitrosourea<sup>14</sup>). In these models, the co-expression has been described only in astrocytes abutting the necrotic areas<sup>13,14</sup>.

We have used immunohistochemical techniques to detect GFAP and VIM on consecutive Epon embedded semithin sections of optic nerves of adult rats to deter-

mine the co-expression of both proteins in normal astrocytes<sup>4</sup>. The thinness of these sections (0.5  $\mu\text{m}$ ) makes it possible to compare the immunostaining for both antigens in consecutive sections of the same cell. In the present study, we apply this method to demonstrate co-expression of GFAP and VIM in reactive astrocytes appearing around a brain stab wound.

Eight Wistar rats aged 4 months were used for our study. Under ether anesthesia, a small incision was made on the scalp. The skull was drilled on a point located 5 mm behind the bregma and 3 mm to the right of the midline. A 3 mm-deep brain wound was made with a sterile needle (1 mm thick) on the right cerebral hemisphere. Animals were killed 5 days after the lesion. Under deep ether anesthesia, rats were perfused with 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Cubic tissue samples of 4–5 mm thick centered by the wound were obtained after the perfusion. The most superficial part of the sample, corresponding to the cerebral cortex, was trimmed out in four 1.5–2 mm-thick tissue blocks bearing the wound and neighbouring brain tissue. Tissue samples obtained from the left hemisphere at the same level of the wound were used as non-lesioned controls. Tissue blocks were embedded in Epon without postfixation in osmium tetroxide, and oriented with the wound track perpendicular to the section plane. Five sets of 10 consecutive semi-thin sections 0.5  $\mu\text{m}$  thick were obtained from each block. Alternative sections were immunostained for GFAP and VIM as described previously<sup>4</sup>.



Figs. 1–6. Figs. 1 and 2: low-power magnification of consecutive semi-thin sections of rat cerebral cortex showing the wound track (asterisk) and the adjacent nervous tissue immunostained for GFAP (Fig. 1) and for VIM (Fig. 2). The positive band is thicker when immunostained for GFAP than for VIM. Magnification 55 $\times$ . Figs. 3 and 4: high-power view of the reactive glial band immunostained for GFAP and VIM. Profiles immunostained for GFAP outweigh those immunostained for VIM. Magnification 120 $\times$ . Figs. 5 and 6: high-power magnification of reactive astrocytes immunostained for GFAP (Fig. 5), and for VIM (Fig. 6) showing the expression of both antigens in consecutive sections of the same cells. Magnification 300 $\times$ .

Reactive astrocytes around the brain wound showed a positive immunostaining for both antigens studied. In both cases, the strongest concentration of immunostained profiles corresponded to the area closest to the lesion, fading beyond this limit (Figs. 1–2). The width of the immunopositive band varies with the antigen studied. Two regions could be distinguished according to their immunoreactive pattern: (i) Inner zone, accounting for the first 300–350  $\mu\text{m}$  from the wound limit, showing a positive immunostaining for both, VIM and GFAP, in reactive astrocytes. (ii) Outer zone, only immunostained for GFAP. The width of this region was considerably greater than that of the inner zone. GFAP<sup>+</sup> astrocytes were frequently found over all the surface of sections. Thus, the border of the outer region seemed to fall beyond the tissue block. Neither GFAP<sup>+</sup> nor VIM<sup>+</sup> astrocytes were seen in the cerebral cortex of the control left hemisphere.

The pattern of immunostaining of inner regions of consecutive sections immunostained for GFAP and VIM was similar, though the amount of GFAP-positive profiles was always larger (Figs. 3–6). To determine the extent of the co-expression phenomena, the somata of immunopositive astrocyte of the inner zone were marked in photographic prints of consecutive tissue sections. Results showed that about 60% of GFAP<sup>+</sup>-reactive astrocytes of the inner zone were also VIM<sup>+</sup>, while the remaining 40% only expressed GFAP. Astrocytes positive for VIM but negative for GFAP were not observed.

According to our results, reactive astrocytes appearing in the cerebral cortex 5 days after a brain stab wound, express GFAP and VIM. The present study was done on gray matter to achieve a more accurate identification of changes in the glial antigen expression of reactive astrocytes. Though detected in some astrocytes of the white matter<sup>4,16,17</sup>, VIM-positive astrocytes have not been described in the cerebral cortex of adult rats. On the other hand, protoplasmic astrocytes of the normal cerebral cortex do not express GFAP<sup>7,9–11</sup>. The study was performed at the 5th postlesional day, which according to several studies accounts for the time of the maximal glial reaction<sup>8–11,20</sup>.

Our results confirm the increase in the expression of GFAP<sup>1,2,8,10,11,15,18–20</sup> and the appearance of positivity for VIM<sup>13,14,19</sup> in reactive astrocytes after the brain injury. The co-expression of GFAP and VIM on reactive astrocytes has been studied in stab-wounded brains of neonatal rats<sup>13</sup> and in the glial reaction around laser in-

duced brain necrosis and around experimental tumours<sup>14</sup>. Both investigations used a double immunostaining on cyrostat or paraffin sections.

In our study, consecutive semi-thin sections 0.5  $\mu\text{m}$  thick have been immunostained alternatively for GFAP and VIM. Our technique allows a great resolution to establish comparisons among the expression of both antigens. According to our results, confirming previous studies<sup>13,14</sup>, the reactivity against VIM is more restricted than for GFAP. An immunopositive band of 300–350  $\mu\text{m}$  thick appears surrounding the wound. In this region, 60% of the astrocytes express both VIM and GFAP, while the remainder is positive only for GFAP. The lesser proportion of VIM-positive astrocytes compared to the population of GFAP-positive astrocytes has been highlighted by other authors<sup>13,14,19</sup>, though not quantified.

The genesis of reactive astrocytes as a result of hypertrophy or hyperplasia is still under discussion. Mitosis has been described in reactive astrocytes surrounding the brain wound<sup>5</sup>. Autoradiography using tritiated thymidine shows labelling of reactive astrocytes<sup>9,11,12</sup>. The co-expression of GFAP and VIM on reactive astrocytes could be interpreted as a sign of immaturity of these cells, indirectly supporting their origin from a recent mitosis. Actually, developing astrocytes in the first two postnatal weeks can co-express both antigens<sup>13,16,21</sup>. However, several facts contradict this idea. Autoradiographic studies with tritiated thymidine demonstrate a low labeling index of reactive astrocytes<sup>9,11,12</sup>. Using the 'cumulative labelling' method, i.e. counting after repetitive injections of tritiated thymidine for 6 days after the brain lesion, only 17% of reactive astrocytes were labelled<sup>11</sup>. All these data suggest that the majority of the reactive astrocytes mainly derive from the hypertrophy of pre-existing astrocytes. Thus, newly formed astrocytes should represent a minority. According to Dahl and Bignami<sup>2</sup>, after a cerebral wound, protoplasmic astrocytes of the gray matter show a considerable increase in the expression of GFAP, becoming fibrous astrocytes. In our material, reactive astrocytes frequently showed hypertrophic somata and thick cell processes, showing a morphology rather resembling fibrous astrocytes of the white matter. The co-expression of GFAP and VIM on reactive astrocytes can also be considered in support of this idea since various studies have demonstrated such co-expression in fibrous astrocytes of the white matter of normal adult animals<sup>4,16,17</sup>.

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