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AGE-DEPENDENT MORPHOLOGICAL CHANGES IN dBcAMP-TREATED ASTROCYTES

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Summary Since changes in cell morphology are conspicuous features of astrocyte reaction, we resorted to an histometric approach to evaluate age influence on such morphological response to activating stimuli. To this end, first subculture of rat brain astrocytes at 1, 9 or 21 days in vitro (DIV) were treated during 2 hs with 1 mM of dBcAMP, a chemical compound known to induce cell differentiation. Following treatment, immunoperoxidase labeling of GFAP, specific marker of astrocyte activation, was carried out. Although total count of GFAP-positive cell foci was greater in treated samples in all times tested, when such cell foci were evaluated by image analysis, differences between perimeter/area ratios of such foci were only statistifically significant at 1 DIV. It may be concluded that while dBcAMP effect is maintained despite astrocyte aging, the morphological pattern of response varies markedly along the observation period.

Key words: aging astrocytes, dBcAMP treatment, image analysis

Activated astrocytes, commonly recognized morphologically by their enlarged size and numerous cytoplasmic processes, and immunocytochemically by the massive accumulation of GFAP-positive filaments, arise following any type of CNS injury, whether physical, chemical, immunological, viral or bacterial[1]. The fact that astroglial responsiveness is maintained in spite of aging, even at times when cell senescence becomes associated with diminished brain functions, is still not understood[2].

Since astrogliosis phenomena on the whole organism may somehow be recapitulated by cell cultures affording the advantages of easy manipulation and feasible control, cultured astrocytes has been extensively used notwithstanding the limitations of such in vitro model. In its connection, cell aging has been shown to lead to a gradual increase in spontaneously differentiated cells with a concomitant drop in their division potential[3]. On the other hand, it is well known that the addition of dibutyryl cyclic AMP (dBcAMP) to the culture medium of immature astrocytes results in their differentiation into reactive-like astrocytes[4], since dBcAMP is capable of inhibiting growth and altering morphology of such cells, thus providing a suitable in vitro counterpart of in situ response to neural injury[5], within the limitations of the currently employed criteria to define the reactive state[6].

With the aim to evaluating age influence on cell response to dBcAMP, we resorted to astroglial cultures obtained from brains of newborn Wistar rats as previously described[7]. After 1, 9 or 21 days in vitro (DIV) of first subculture in coverslips of Leighton tubes, cell monolayers were treated with 1 mM dBcAMP in PBS during 2 hs at 37C. Treated samples, as well as matched controls incubated in PBS alone, were harvested, washed 3 times with 0.05 M Tris-saline buffer, and fixed with chilled acid methanol for 20 min at -20C. GFAP labeling was then carried out by the peroxidase-antiperoxidase (PAP method). Accordingly, antisera of rabbit anti-GFAP (Sigma Lab, St Louis, MO, USA) at 1/1600 dilution, goat anti-rabbit (Pell-Freez Biological, Tustin, CA, USA) at 1/200 and PAP-rabbit (Cappel, Cochranville, PA, USA) at 1/200 were consecutively

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employed. Potential sources of variation in peroxidase development were ruled out by normalized conditions: 0.03% diaminobenzidine tetrahydrochloride (Fluka Labs, Hauppage, NY, USA) plus 0.02% hydrogen peroxide for 10 min, followed by washing with tap water. To evaluate morphological changes in treated cultures vs controls, a suitable software (IMAGE PRO-PLUS 1.1, Media Cybernetics, Silver Spring, MD, USA) was employed. Hardware consisted of a black and white CCD video camera coupled to a Zeiss microscope. Under standardized conditions using a 500/510 nm green filter, the perimeter and area of 20 randomly selected GFAP-positive foci were determined for each sample. Recorded values were entered into an standard spreadsheet in order to calculate means and standard deviations (SD). Regarding two or more stained astrocytes as a labeled focus, such foci were counted in randomly selected microscopic fields at 12x final magnification. Student t test was used for statistical analysis taking $p < 0.05$ as significant. As regards histometric analysis of each positive focus, since individual cell outlines could not clearly be discerned, we resorted to an alternative approach, taking the ratio between perimeter and area of each selected focus. In the case of single GFAP-positive cells, we were able to evaluate growth of astroglial processes by means of a graticule of 13 concentric circles, the first being the most proximal and the 13th the most distal. This graticule was superimposed on the image of isolated GFAP-immunolabed cells visualized on the monitor screen. The perikaryon of each selected stained cell was placed in the center of the graticule and all the intersections produced by stained processes with circles were counted. For each sample, the first 15 single labeled cells to be encountered were measured and data analyzed using a two-way ANOVA.

In this study, we evaluated effects of astrocyte aging on the differentiation-inducing properties of dBcAMP, as expressed by the morphological changes taking place in GFAP-positive cells. Mean total focus count ± SEM was significantly greater in treated cultures ($p < 0.01$) than in controls at 1 DIV (8 ± 0.9 vs 5 ± 0.6), 9 DIV (10 ± 0.7 vs 6 ± 0.4) and 21 DIV (5 ± 0.4 vs 3 ± 0.4).

As shown in Table 1, the perimeter/area ratio was only significantly greater in treated vs control samples ($p < 0.05$) at 1 DIV, while at longer times tested, differences failed to reach statistical significance. The increase in perimeter/area ratio recorded in treated cultures at 1 DIV, seems due to cell body retraction rather than changes in focus perimeter, since the major changes took place in area alone. The trend towards significance suggested by values recorded at 21 DIV was disregarded, since by that time controls had shown some degree of cell degeneration as evidenced by intracytoplasmic granulation accompanied by an increase in cell detritus.

Although cultures of lower density than employed herein would have allowed a more accurate delimitation of the cells making up GFAP-positive foci, it should not be overlooked that cells grown at high and low densities exhibited distinct morphologies[8], and such culture modality may markedly affect astrocyte properties, among them cell response to dBcAMP[9]. In this connection, when single GFAP-positive cells were evaluated resorting to the graticule and by a two-way ANOVA (Fig. 1), at all times assayed the single labeled astrocytes from treated samples invariably displayed a greater number of cell processes in-

TABLE 1.— *Morphological changes of GFAP-positive foci in dBcAMP treated vs untreated astroglial cultures.*

Days in vitro	Perim./Nucleus ratio		Area/Nucleus ratio		Perim./Area ratio	
	control	dBcAMP	control	dBcAMP	control	dBcAMP
1	51 ± 31	63 ± 66	518 ± 464	275 ± 124*	0.104 ± 0.1	0.204 ± 0.1*
9	57 ± 37	110 ± 304	781 ± 1005	1535 ± 2874	0.122 ± 0.1	0.210 ± 0.4
21	70 ± 53	185 ± 377	749 ± 686	1016 ± 398	0.097 ± 0.1	0.161 ± 0.3

* Significant differences ($p < 0.05$) between ratios calculated from mean values (± SD).

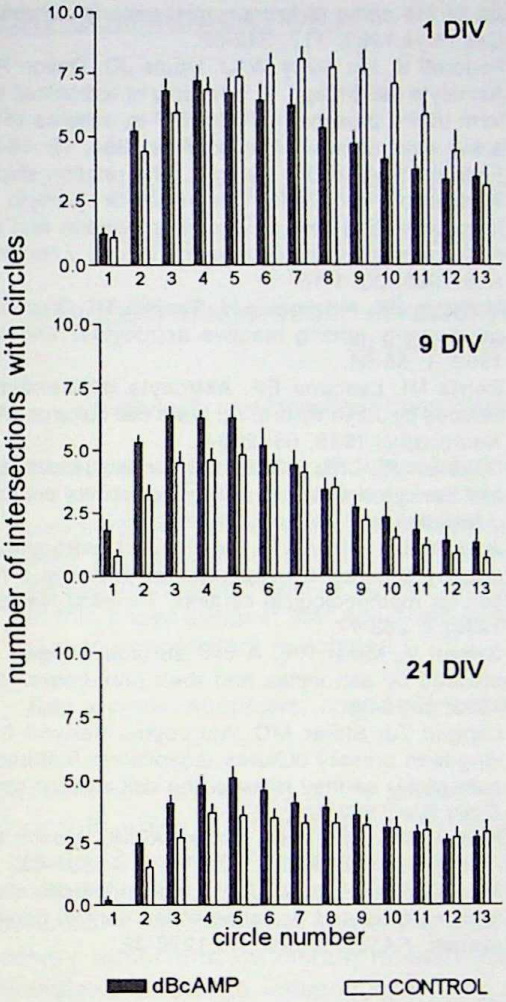

Fig. 1. Vertical bars depict the number of intersections of single GFAP-positive astrocytes with concentric circles (from 1 the most proximal to 13 the most distal) of the test grid used to evaluate cell morphology. Each determination corresponds to 15 single labeled cells to be encountered. DIV: days in vitro.

tersecting with concentric graticule circles 2 and 3, since the first was disregarded because on occasion it was occupied by the cell body itself. Interestingly enough, cell process branching as depicted by intersections with circles 4 to 10 was greater in controls at 1 DIV but not at later times. It should be pointed out that in treated samples, particularly at 1 DIV, most cell processes intersecting with circles 2-3 readily reached and even surpassed the outer circle.

On the basis of results achieved, cell aging failed to exert inhibitory effect on astrocyte response to dBcAMP, as disclosed by the number of GFAP-positive cell foci, although the perimeter/area ratio of such foci was significantly greater at 1 DIV alone. As regards single labeled cells from treated cultures, their morphological pattern was also significantly different at 1 DIV, since by this time dBcAMP had induced longer and more numerous cell processes but less branching. It may be concluded that while dBcAMP effect is maintained despite astrocyte aging, the morphological pattern of response varies markedly.

Although GFAP expression has proven reduced in many long-term cultures to the extent that their identification becomes difficult[10], the capability of such aged cells to re-enter the division cycle[11], suggests that cultured astrocytes may undergo a transition in a manner partly resembling reactive astrogliosis in intact brain. Abundant evidence indicates that, in the aging whole organism, the neuroglial proliferative ability arises in a previously quiescent population of cells which may not have divided for prolonged periods of time and which display few markers of cytodifferentiation [for references see[2, 11]].

However, there are also examples of astrocyte reaction solely disclosed by an hypertrophy occurring in absence of cell division[12]. At any rate, signals which trigger aged astrocyte activation remain to be elucidated. In this sense, the in vitro system may represent a valid tool to discern the stereotypic events involved in the aging process. Thus, the long-term astroglial culture becomes a useful substrate to develop procedures intended to speed up, slow down or arrest a glial reaction which, in the whole organism, may behave as beneficial or detrimental depending on the timing[13].

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Resumen

Cambios morfológicos dependientes de la edad en astrocitos tratados con dBcAMP

Dado que los cambios morfológicos son componente mensurable de la reacción astrocitaria, se recurrió a un procedimiento histométrico para evaluar la influencia de la edad celular en la respues-

ta a los estímulos diferenciadores. Así, subcultivos astrocitarios derivados de encéfalo de rata fueron tratados a los días 1, 9 y 21 de edad con dBcAMP, un compuesto de reconocida acción estimulante sobre ese tipo celular. Consecutivamente a la marcación por inmunoperoxidasa de la proteína gliofibrilar ácida (GFAP), que es marcador específico del astrocito activado, por análisis de imagen se determinó el perímetro y área de los focos celulares positivos. Aunque en todos los tiempos considerados el número total de focos GFAP-positivos fue mayor en los cultivos tratados, la relación perímetro/área de dichos focos sólo arrojó diferencias significativas al día 1 de desarrollo in vitro. Se concluye que, si bien la acción diferenciadora se mantuvo pese al envejecimiento astrocitario, el perfil morfológico de la respuesta celular varió marcadamente a lo largo del período de observación.

References

1. Eng LF. Regulation of glial intermediate filaments in astrogliosis. *In:* Norenberg MD, Hertz L, Schusboe A, (eds). The biochemical pathology of astrocytes. New York: Alan R Liss, 1988; 79-90.
2. Kozlova M, Kentroti S, Vernadakis A. Influence of culture substrata on the differentiation of advanced passage glial cells in culture from aged mouse cerebral hemispheres. *Int J Devl Neurosc* 1993; 11: 513-9.
3. Ponten J, Stein WD, Shall S. A quantitative analysis of the aging of human glial cells in culture. *J Cell Phys* 1983; 117: 342-52.
4. Fedoroff S, Mc Auley WAJ, Houle JD, Devon RM. Astrocyte cell lineage. V. Similarity of astrocytes that form in the presence of dBcAMP in cultures to reactive astrocytes. *J Neurosci Res* 1984; 12: 15-27.
5. Fedoroff S, Ahmed I, Wang E. The relation ship of expression of statin, the nuclear protein of nonproliferating cells to the differentiation and cell cycle of astroglia in cultures and in situ. *J Neurosci Res* 1990; 26: 1-15.
6. Malhotra SK, Aldskoguis H, Shnitka TK. Biochemical diversity among reactive astrocytes. *Dendron* 1992; 1: 55-68.
7. Berría MI, Lascano EF. Astrocyte differentiation induced by Junin virus in rat brain cell cultures. *Acta Neuropathol* 1985; 66: 233-8.
8. Goldman JE, Chiu F-C. Growth kinetics, cell shape, and the cytoskeleton of primary astrocyte cultures. *J Neurochem* 1984; 42: 175-84.
9. Juurlink BHJ, Hertz L. Plasticity of astrocytes in primary cultures: an experimental tool and a reason for methodological caution. *Develop Neurosci* 1985; 7: 263-77.
10. Szigeti V, Miller RH. A cell surface antigen expressed by astrocytes and their precursors. *Glia* 1993; 8: 20-32.
11. Langan TJ, Slater MC. Astrocytes derived from long-term primary cultures recapitulate features of astrogliosis as they re-enter the cell division cycle. *Brain Res* 1992; 577: 200-9.
12. Hatten ME, Liem RHK, Shelanski ML, Mason CA. Astroglia in CNS injury. *Glia* 1991; 4: 233-43.
13. Mucke L, Eddleston M. Astrocytes in infectious and immune-mediated diseases of the central nervous system. *FASEB J* 1993; 7: 1226-32.

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On résiste à l'invasion des armées mais on ne résiste pas a l'invasion des idées.

Se resiste a la invasión de los ejércitos pero no se resiste a la invasión de las ideas.

Víctor Hugo (1802-1885)

Histoire d'un crime, 1852