ANAPLASIA AND HETEROGENEITY OF GFAP EXPRESSION IN GLIOMAS

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GFAP (glial fibrillary acidic protein) distribution was investigated in selected areas of glioblastomas and astrocytomas. The proliferating cell population of glioblastomas was GFAP negative and contained many mitoses which were also negative. The old, deeply located areas were composed of cells with visible cytoplasm, intensely GFAP-positive; mitoses in these areas were both GFAP-positive and negative. GFAP-positive reactive astrocytes, once trapped in the tumor, were no longer distinguishable from positive tumor cells. They sometimes contained mitoses. In astrocytoma, anaplasia was due to the development of a GFAP-negative population with negative mitoses. The problem of dedifferentiation and differentiation of malignant gliomas is discussed taking into account the possibility that malignancy may be due to increasing mutation rates of tumors. The problem of redifferentiation of already dedifferentiated cells is also discussed.

Anaplasia in gliomas may be conceived as due to heterogeneity of tumor cell populations. The heterogeneity has been hypothesized from karyotypic analyses (26, 27, 37), cytophotometric measurements of DNA (9, 23), flow cytometry (19, 20) and comparisons of established human glioma cell lines (3). Whether it is a phenotypic feature or an expression of genotypic variability, due to the progressive increase in mutation rates of tumor populations (29), is still debated (2, 30).

The manifestations of heterogeneity are numerous and concern many cell properties, including the expression of glial fibrillary acidic protein (GFAP) (22). The distribution of GFAP in gliomas has been extensively studied in recent years (4, 7) and the main conclusions are that all tumors deriving from cells with gliofibrillogenetic capacity are GFAP-positive and that the number of positive cells is inversely related to the degree of anaplasia. In particular, as was to be expected, the small, less mature cells of gliomas, which proliferate more rapidly (18) and are responsible for tumor invasiveness and growth (15), are GFAP-negative (42).

The most important problem deriving from heterogeneity of tumor cell populations is the relationship between heterogeneity and tumor growth kinetics. It has been demonstrated that the crucial point is not the change in the cell cycle time, but rather that of the growth fraction (18, 38) since with the increasing malignancy of the tumor, cells are recruited from the non-proliferating to the proliferating pool and clonogenic cell populations develop. The new cell populations will have an enhanced proliferating capacity and will coexist with the old ones, so that the phenotypic feature of a tumor district will depend on the stage in which the tumor is morphologically examined (36) as well as on the action of known and unknown epigenetic factors.

We re-evaluated the distribution of GFAP in morphologically different districts of astrocytic gliomas in light of the above expressed concepts.

Material and methods

Twenty-one astrocytic gliomas were selected (according to the presence of the areas mentioned below) out of our collection of tumors studied for GFAP: 6 astrocytomas, 6 anaplastic astrocytomas

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and 9 glioblastomas. Specimens from surgical biopsies were fixed in Carnoy at 0-4 °C, embedded in paraffin and cut in 7-micron thick sections. The following histologic methods were used: hematoxylin-eosin, and Luxol fast blue B-PAS-hematoxylin for vessels. GFAP was demonstrated by the peroxidase-antiperoxidase (PAP) method, utilizing anti-GFAP serum (DAKO Corp., Santa Barbara, Calif., U.S.A.), diluted 1:600 in phosphate-buffered saline. Normal swine serum, swine antirabbit immunoglobulins and the PAP complex were from DAKO. Parallely, monoclonal antibody (Labsystems, Copenhagen, Denmark), diluted 1:200, was used with the avidin-biotin peroxidase complex method (ABC Kit, Vector Laboratories, Burlingame, Calif., U.S.A.).

The following tumor areas were considered: astrocytoma areas (fibrillary, protoplasmic and gemistocytic); astrocytoma areas with different degrees of anaplasia; glioblastoma areas (proliferative, invasive [towards the cortex and the white matter], central or perinecrotic, peripheral and reactive).

Mitoses were accurately investigated and classified according to the positive or negative reaction for GFAP in the cytoplasm.

Fig. 1 - Glioblastoma. a) Proliferative area: elongated cells, mostly with isomorphic nuclei and a circumscribed necrosis with pseudo-palisade. Negative reaction for GFAP, 200 x; b) Proliferative area: GFAP-negative cells with polymorphic nuclei and many mitoses, 200 x; c) Central area: most cells are GFAP-positive, 300 x; d) Mitosis in a GFAP-positive cell, 400 x.
Results

Glioblastoma

a) Proliferative areas were characterized by a high cell density and small cells. These showed either isomorphic normochromatic or polymorphic nuclei. The cytoplasms were usually unrecognizable or bipolar; only a few cells showed more developed cytoplasms. Many capillaries, endothelial proliferations and buds, as well as circumscribed necroses with pseudo-palisading and abundant mitoses were present. GFAP: most cells were negative (fig. 1a). Mitoses were negative (fig. 1b).

b) Central, deeply located areas often bordered extensive coagulative necroses. Most cells showed large cytoplasms, spindle shaped or with a gemistocytic aspect, and polymorphic nuclei. Vessels were usually large, with pathologic walls and thromboses. A few cells resembled those of areas a). Only a few mitoses were recognizable. GFAP: most cells showed positive cytoplasms and short processes (fig. 1c). Mitoses were either po-

Fig. 2 - Glioblastoma. a) Invasive cortical area: infiltrating negative cells and positive reactive astrocytes, GFAP, 200 X; b) Negative mitosis among positive reactive astrocytes, GFAP, 400 X; c) Positive reactive astrocytes in a negative proliferating cell population, GFAP, 300 X; d) Invasive area in the white matter with positive reactive astrocytes and negative tumor cells and mitoses, GFAP, 300 X.
sitive (fig. 1d) or negative. The positive ones were often large and atypical.

**c) Invasive cortical areas.** In addition to neurons and reactive astrocytes, small cells with normo- or hyperchromatic nuclei could be recognized. Endothelial proliferations, buds and vascular glomeruli were present. Mitoses were abundant. GFAP: small cells were negative (fig. 2a). Reactive astrocytes were intensely positive in the cytoplasms and processes. Most mitoses were negative (fig. 2b); however, some mitoses could be identified in large positive cytoplasms of reactive astrocytes.

**d) Invasive areas in the white matter.** Reactive astrocytes were numerous and oriented along myelin fibers: they were intermingled with small cells with normo- or hyperchromatic nuclei (fig. 2c-d). Many mitoses were present. GFAP: reactive astrocytes were positive as in c). Small cells were negative. Most mitoses were negative, with the exception of some in large, positive cytoplasms, also with processes.

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*Fig. 3 - Glioblastoma. a) Transition area with positive reactive astrocytes, also with mitosis (thick arrow) and negative tumor cells and mitoses (thin arrow), GFAP, 400 X; b) Transition area: positive reactive astrocytes are indistinguishable from positive tumor astrocytes. Tumor cells and mitoses are negative. GFAP, 400 X; c) Transition area: mitosis in an intensely positive cell, GFAP, 1000 X; d) Circumscribed necrosis with pseudo-palisades: positive reactive astrocytes, also within the necrosis, GFAP, 300 X.*
e) *Transition areas from c) and d) to a)* were characterized by an increasing number of small cells with normo- or hyperchromatic nuclei and mitoses. Reactive astrocytes were decreased in number. GFAP: Small cells were negative. Reactive astrocytes were positive with many processes; however, as they became more deeply located they lost the processes so that they were indistinguishable from tumor astrocytes with visible cytoplasm (fig. 3a-b). Some of these cells still showed one large and vesicular nucleus, whereas others were polynucleated. Negative mitoses were greatly increased in number. Positive mitoses were present.

f) *Areas with circumscribed necroses.* These structures could be found in proliferative, invasive, and in central areas. In the proliferative areas they were surrounded by pseudo-palisades; in the central areas pseudo-palisades were sometimes absent. In necroses with pseudo-palisades, mitoses were abundant and occurred mostly outside of the pseudo-palisades which, in contrast, were rich in hyperchromatic, round, lymphocyte-like nuclei. GFAP: in necroses with pseudo-palisades cells and mitoses were negative. In necroses without pseudo-palisades, cells were mostly positive, and positive material from damaged cytoplasms and processes inside the necrosis was found. When circumscribed necroses with pseudo-palisades occurred in areas such as e), many large reactive or transitional astrocytes were found both outside and inside the necrosis or within the pseudo-palisade (fig. 3d). Those located inside showed a damaged and regressive aspect. Astrocytes were randomly distributed and did not accumulate around the necroses.

**Astrocytoma**

* g) Typical areas showed low cell density, fibrillary, protoplasmic and gemistocytic aspects, and a few small vessels. Mitoses were absent. GFAP: positive reaction was found as a network in fibrillary areas and in most cytoplasms in protoplasmic and gemistocytic areas (fig. 4a).

* h) Areas with initial anaplasia showed an increased cell density and mitoses. No vessel modification was observed. GFAP: there were negative small cells. Mitoses were negative.

* i) Frankly anaplastic areas showed high cell density, nuclear polymorphism, and many mitoses. Vessels were increased in number and size and showed endothelial proliferations. GFAP: most cells as well as mitoses were negative (fig. 4b).

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**Fig. 4** - a) Astrocytoma. Protoplasmic cells intensely GFAP-positive, 300×; b) Anaplastic astrocytoma: development of a negative population with negative mitoses, GFAP, 300×.
Discussion

Before discussing the significance of the distribution of GFAP, a short comment on mitoses is necessary. We considered their frequency as representative of the proliferating capacity of the cells subpopulations to which they belonged. This inference is only partially correct, since many factors may influence the demonstration of mitoses in histologic sections, such as anoxia, delayed fixation (5, 6), and type of fixation. However, the number of mitoses can be considered as representative of the proliferative activity of a given cell population, since in our material all mitoses were subjected to the same influencing factors.

Secondly, we utilized the expression of GFAP during mitosis to establish what type of tumor cell population the mitosis belonged. Some reports seem to support this deduction. In developing brains, cells of the germinal zone, committed to differentiate along the glial line, acquire the capacity of GFAP expression very early, long before the loss of proliferative activity, and GFAP expression is preserved during mitosis (24, 40). This property has been verified by us in developing rat brain as well as by others in cultures of glial cells, in which the only site of the cytoplasm without GFAP during mitosis was that of the spindle (13).

From our observations it can be deduced that both anaplasia in differentiated astrocytomas and active proliferation in glioblastomas are shown by the presence of cell populations characterized by the paucity of cytoplasm, the absence of GFAP, and the presence of many GFAP-negative mitoses. The negative correlation between cell density and GFAP immunoreactivity already described (42) is thus confirmed, because the increasing cell density is due to the new cell population. Many authors agree on this finding (8, 12, 21, 41). In dedifferentiating astrocytomas, all the mitoses were GFAP-negative and belonged to the new cell population. Mitoses were not observed in well differentiated astrocytomas, so that we could not ascertain whether they belonged to a GFAP-positive or GFAP-negative population.

In glioblastomas, our findings confirm the GFAP-negative reaction of small cells (42) and that the small cells are responsible for invasiveness and growth (15). They indicate that the two cell populations, with and without cytoplasms expressing GFAP, have two different kinetics, as the relative amount of GFAP-positive and negative mitoses point out. Moreover, it seems, that the fast-growing cell population originates from the slow-growing one, as shown by the presence of areas with a mixed cell population. The morphologic composition of the different districts of glioblastoma seems to be constantly due to modulation of the two aforementioned cell populations.

Starting from the principle that an increase in the growth fraction, which accompanies the malignant transformation of gliomas, is due to the recruitment of cells from the nonproliferating to the proliferating pool, it remains to be established whether this is due to new mutations or simply due to the action of epigenetic factors. In the first case, the major astrocytic characteristic of glioblastomas, i.e., GFAP expression, should eventually disappear after repeated mutations. Actually, since the median survival of glioblastomas is rather short, we do not know if this is true; the cases of glioblastomas with a very long duration cannot be taken into consideration, because they showed, at least morphologically, peculiarities which differentiated them from typical glioblastomas (16, 17). However, in the second case, the morphologic aspect of the different districts of glioblastoma might be the result of a modulation controlled by epigenetic factors, so that the loss of GFAP expression might be reversed, i.e., astrocytic differentiation with the appearance of GFAP in undifferentiated cells. There is a number of instances of factors inducing differentiation of malignant glia, for example, dbcAMP; in nonglial tumors, such as neuroblastoma, even the reversal of neoplasia may result (14, 43). All these possibilities have already been extensively discussed (11) and should be kept in mind. In this regard, it should be stressed that, in spite of much debate, the old and unresolved problem of whether astrocytomatos areas of glioblastomas are the remnants of an astrocytoma which transformed into a glioblastoma or the product of differentiation of glioblastoma cells (25, 31, 32, 35) has not found a definite answer. We are more inclined to believe in the first hypothesis, because at the beginning anaplasia is mostly a circumscribed phenomenon, so that the possibility that an astrocytoma, even after its malignant transformation, continues to grow as an astrocytoma in untransformed parts should not be underestimated.

Another interesting point of discussion is the destiny of reactive astrocytes once they are trapped in the advancing tumor proliferation. They lose their processes and are no longer distinguishable from tumor astrocytes, as already pointed out (1). Perhaps they contribute to the
morphology of peripheral areas of glioblastomas to the point of giving an astrocytic aspect to a proliferative area composed mainly of undifferentiated cells. If circumscribed necroses with pseudo-palisades develop in the proliferative areas, reactive astrocytes seem to react passively and their spatial distribution in the area remains unchanged. Even though reactive astrocytes seem to be partly involved in the tumor process from the immunologic point of view (10), there is no direct evidence that they participate actively in the cell composition of the tumor. In experimental brain tumors induced transplacentally by ethynitrosourea, strongly GFAP-positive reactive astrocytes are abundant in early tumoral lesions, in peritumoral tissue and in the peripheral areas of fully developed tumors; however, they eventually disappear from the central tumor and do not seem to be involved in tumor growth (28).

The relationship between reactive astrocytes and malignant gliomas has not yet been completely clarified and deserves much more attention. Reactive astrocytes show mitoses and should therefore be regarded as a slowly growing population in contact with an active proliferating tumor population. A possible reciprocal influence cannot be ruled out. There are other examples of different cell populations which grow together, for instance in gliosarcomas. On the basis of experimental data, the hypothesis of a horizontal transmission of malignancy (transfection) from malignant glial cells to putative normal differentiated stroma cells has been considered (30).

Finally, circumscribed necroses with pseudo-palisades should be kept separate from circumscribed necroses without pseudo-palisades. The former should be regarded as a characteristic sign of active growth (34). They develop from actively proliferating centers of the tumor as the final product of mitotic imbalance (44), since the mean generation time of endothelial cells is longer than that of tumor cells (39). The pseudo-palisades are composed of cells belonging to the new actively proliferating population, which is GFAP-negative. In fact, they are rich in mitoses, whereas the necroses contain pyknotic nuclei. This material is probably composed of denaturated DNA, and represents the final product of abortive mitoses. This interpretation is given on the basis of observations in fluorescence microscopy after fluorochromization in medulloblastomas (33).

Anaplasia ed eterogeneità dell'espressione della GFAP nei glioni

È stata studiata la distribuzione immunoistochemica della proteina acidica glicofibrillare (GFAP) in aree selezionate di glioblastomi ed astrocitomi. L'espressione della GFAP varia a seconda del tipo e localizzazione delle popolazioni cellulari: quella proliferante del glioblastoma e quella anaplastica degli astrocitomi sono negative. Pure negative sono le mitosi relative. Le aree situate in profondità sono costituite prevalentemente da cellule a citoplasma evidente, intensamente positivo per la GFAP, con mitosi sia GFAP-positive che negative. Gli astroidi reattivi GFAP-positivi, quando sono inclusi nella proliferazione tumorale, sono indistinguibili dagli storici tumorali GFAP-positivi, anche per la positività delle loro mitosi. Il problema della dedifferenziazione cellulare dei glioni maligni è discusso considerando la possibilità che la malignizzazione sia dovuta all'aumento del grado di mutazione del tumore, con comparsa di nuove popolazioni cellulari a cinetica accelerata. Quello della differenziazione invece non può essere disgiunto dalla questione degli astroidi reatiivi inclusi nel tumore.

References


