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DNA Flow - Cytometry of Brain Tumours - A Preliminary Study

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Abstract

The technique of flow-cytometry has been used to measure the cellular DNA content of human brain tumours using 4,6 -Diamidino-2-phenyl-indolehydrochloride (DAPI) as fluorochrome and chicken erythrocytes as internal standards. Results of this preliminary study on 64 brain tumours show that the DNA index of brain tumours is widely distributed (1.4 to 3.5) and the percentage proliferating fraction in tumours is significantly higher (22.0 $\pm$ 9) than the normal brain issues (8.3 $\pm$ 0.5). Multiple DNA indices were observed in some cases indicating the presence of different sub-populations of cells. Most of these were malignant tumours. An attempt to analyse the tumours taking both DNA index and percentage proliferating fraction together, indicate that such measurements could be useful in distinguishing more clearly between the malignant and benign tumours.

Key words -

Flow-cytometry,
Brain tumours,
DAPI,
Chicken erythrocytes,
DNA index,
Percentage proliferating fraction,
Malignancy

Distinction between benign and malignant neoplasms, on the basis of histological investigations alone is often difficult, particularly in tumours of the central nervous system. Therefore, it is desirable to compliment the clinical and histological examination of brain tumours with studies which may provide quantitative and objective information relating to the biological behaviour of the tumours.

The importance of quantitative measurements of the cellular DNA contents in tumours is well established [1], [2], [3]. The cellular DNA contents of brain tumours as measured by the conventional biochemical techniques have been reported to be higher than the normal brain tissue [4]. Cytophotometric [5], [6] studies have confirmed these results. However,

methodological difficulties in making accurate measurements on a larger number of cells, have limited the application of these techniques. With the advent of flow-cytometry [7], [8] these problems have been overcome as it allows rapid and precise measurements on a large number of cells. When stained with DNA specific fluorochromes, a frequency distribution of DNA content in the cell population can be easily obtained with this technique. DNA flow-cytogram provides information on the distribution of cells in the various phases of the cell-cycle and permits calculation of the mean DNA content per cell and DNA content dispersion in the population [9]. Preliminary results on a study of human brain tumours (64 cases) using this technique are reported here.

Material and Methods

Tumor biopsies from the Neuro-surgery department, were collected immediately after surgery in cold minimum essential medium (MEM 199). The biopsy material was divided into a few representative areas (2 to 4 depending on the sample available) and minced using fine scissors and scalpel blade in cold Hanks' balanced salt solution (HBSS).This preparation was then passed through pasteur pipette a few times and filtered using a fine nylon mesh to remove clumps. The cells thus obtained were washed in cold HBSS, fixed in 70 percent ethanol and stored at 4 degree C. Before measurements, the cells were washed in distilled water to remove ethanol and stained with DAPI, (Serva, Heidelberg) as described earlier [10]. Briefly, cells were treated (20 min) with 0.22 M citric acid buffer (pH 2.1) containing between 20 detergent and subsequently stained with DAPI (5μ M), present in 0.4 M sodium phosphate buffer (pH 8.9). The measurements were made on an ICP-22 flow-cytometer (PHYWE) equipped with a multichannel analyser (Canberra 8100). The flow-rate was controlled using a syringe pump. Chicken erythrocytes stained identically and measured along with the samples served as internal standards. Routine histological examinations of the biopsy specimen (paraffin sections, stained with Haematoxyline and Eosin) were also carried out. The classification and grading of tumours were based on morphologically identifiable cell types and histological pattern as suggested by Zulch [11].

Results and Discussion

The DNA histograms (DNA content vs cell number) of unstimulated human peripheral blood leukocytes (PBL), normal brain and brain tumours are shown in Fig. 1. Peripheral blood leukocytes always gave one major peak (Fig. 1A) for diploid cells (2c) corresponding to G0/G1-phase of the cell-cycle. The DNA content of cells increases during the DNA synthetic (S) phase to double the amount (4c, second peak) and returns to the diploid amount after mitosis. PBL are normally non-proliferating; approximately 96 per cent of the cells were observed to be in the G0/G1-phase of the cell-cycle. The percentage of cells in S-and G2+M-phases, together called the proliferating fraction (PF), amounted to only 4 per cent (Table I). Since there is hardly any contribution from the S-phase to the proliferating fraction (Fig 1A), the second peak could also be due to clumps, chance clincidences during measurement or some polymorphs which are known to have tetraploid DNA content. For obtaining the flow-cytograms of normal brain tissue, cells from different areas of the brain were taken. All these gave a distinct diploid peak similar to leukocytes (Fig. 1B), but the PF was 8.3 ± 0.5 per cent with practically no cells in the S-phase (Table I). It is interesting to note in this connection that some types of glia cells in the human brain have been observed to have tetraploid. DNA content [12]. In

contrast, the pattern of histograms for brain tumours broadly fell into two categories. The first category demonstrated a distinct, single diploid peak with cells in all the phases of the cell-cycle (Fig. 1C). PF reported here was calculated from this category of samples only, the average value being 22 ± 9 (Table I), significantly higher than that of normal brain tissue. The second category showed multiple Go/G1 peaks indicating the presence of different subpopulations with varying DNA contents (Fig. 1D).

DNA flowcytograms of peripheral blood leukocytes (PBL) (A), normal brain (B), low grade astrocytoma (C) and high grade astrocytoma (D)

Table I - Average values of DNA Index and Percentage Proliferating Fraction of PBL, Normal Brain and Brain Tumours

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Figure 2 shows the values of proliferating fractions in PBL, normal brain and brain tumours (benign and malignant). The malignant tumours consisted of grade III-IV gliomas (22), glioblastoma multiforme (2), medulloblastoma (5), malignant ependymoma (1) and secondaries (3). The benign tumours include, meningioma (10), schwanoma (7), pituitary adenoma (3), neurofibroma (1), ependymoma (1) and Grade I-II gliomas (9). It should be noted that in case of malignant tumours not only the PF is high (25.8) as compared to benign tumours (19.4) but the range is also larger. The distribution of PF values observed in brain tumours is given in Fig. 3 which shows that most of the tumours have a PF in the range of 15 to 30 percent.

Percentage proliferating fraction (PF) of PBL (A), Normal brain tissue (B), Brain Tumours [all (C), Benign (D) and Malignant (E)]

Frequency distribution of % PF in brain tumours

Histograms of PBL and a brain tumor with chicken erythrocytes used as internal standards are shown in Fig. 4a. The use of an internal standard minimises errors due to variations in staining and instrumental fluctuations during measurements, allowing a more precise determination of DNA content and hence the DNA index. DNA index is defined relative to the DNA content of normal cells (unstimulated PBL were taken as reference for diploidy with DNA index=2) and is calculated as-

DNA Index= DNA content of the sample /DNA content of PBL × 2 = PS / PCE ÷ PPBL / PCE × 2

where PS. PPBL and PCE are the modal channel numbers corresponding to G0/G1- peak of the tumor sample, PBL and chicken erythrocytes respectively.

DNA flowcytograms of PBL and a brain tumor (meningioma) with internal standard (chicken erythrocytes). For details see text

The average values of the DNA index of PBL, normal brain and brain tumours are given in Table I, and the DNA index distribution for brain tumours is shown in Fig. 4b. It is seen that the index varies from 1.4 to 3.7 with many points clustered around 2.0. This shows that although majority of the brain tumours have a near diploid DNA content, hypo and hyper diploidy is not uncommon. This is an agreement with earlier results of flow-cytometry [13] and studies on the chromosomal abnormalities in brain Ttmours, wherein variations in chromosome numbers were observed in both malignant and benign tumours, but the quantitative differences were reported to be larger in case of malignant brain tumours [14], [15].

Frequency distribution of the DNA index observed in brain tumours

The tumor flow-cytograms also showed incidences of brain tumours with multiple DNA indices (9

cases) indicating more than one population of cells. Most of these were found to be a malignant tumours (5 malignant astrocytomas, 2 secondaries and 1 medulloblastoma with meningial infiltration) with the exception of a meningioma which was classified both clinically and histologically as benign. Flow-cytogram of this meningioma (Fig. 4) reveals multiple DNA indices (2.07 and 2.44). Examination of the histological sections also suggested the presence of two types of cells, one with small nucleus and the other with larger nucleus. Occassional incidence of meningiomas with dual DNA indices as revealed by flow-cytometry has also been reported by Frederksen [16] and aneuploid meningiomas without histological malignant features have been demonstrated by chromosomal studies [17]. Benign meningiomas occassionally show bizarre hyperchromatic giant nuclei as in the present case which might raise the suspicion of malignancy. However, as these are found in the absence of rapid growth, increased mitotic activity or invasion, such tumours are considered to be benign [18]. It will be interesting to have the follow-up study of this patient.

Flowcytogram and photomicrograph of a meningioma

The pattern of flow-cytograms obtained from different areas of the same tumor biopsy sometimes gave quantitatively different pattern indicating heterogeneous nature of tumours. One example of flow-cytograms obtained from different areas of a grade IV astrocytoma is presented in Fig. 6. It appears that there are three cell population in this case having DNA indices 2.04, 2.23 and 3.57. The relative proportions of these sub-populations in different areas are quantitatively different. The histological heterogeneity in malignant brain tumours, specially in astrocytomas is well known [19]. Previous flow-cytometric studies have also shown that malignant gliomas are generally associated with a high degree of heterogeneity with respect to ploidy. In contrast, metastaic brain tumours are more homogeneous [20]. Further analysis of heterogeneity should be useful as it is known to influence the biological behaviour of the tumours.

Flowcytogram obtained from different areas of a grade IV astrocytoma

In previous flow-cytometric studies on brain tumours [13], [16], [20], [21], [22], [23] the DNA content was not measured precisely. In some of the studies, correlation of only one index viz., the modal DNA content (Ploidy) or proliferating fraction was attempted with the state of the tumor. In the present studies, we have evaluated the DNA index more accurately with the help of an internal standard. Our studies suggest that the simultaneous analysis of more than one parameter viz., DNA index, percentage proliferating fraction and degree of heterogeneity together could give a better insight into the state and biological behaviour of the tumor. An initial analysis of this nature indicate that it could be useful in distinguishing clearly between malignant and benign tumours. Therefore, further studies to evaluate the contributions of flow-cytometry in improving diagnosis, treatment and prognosis of brain tumours are warranted.

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