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The Applications of the BrdUrd-Technique for the Estimation of Cycling S-Phase Cells in Human Renal Cell Carcinoma

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Summary. After testing the BrdUrd technique on experimental tumour cell lines, we applied the technique to human renal cell carcinoma in vitro. We compared the results with the data acquired after FCM analysis and 3H-thymidine treatment. In contrast to BrdUrd the 3H-thymidine uptake seemed to be limited in suspended cells. FCM data represented the DNA distribution of cells. BrdUrd labelling on the other hand detected DNA synthesizing cells. Only both methods in parallel were able to discriminate between proliferating cells and resting cells with an S-phase equivalent DNA content.

Key words: Proliferation of renal cell carcinoma – Flow cytometry – BrdUrd-labelling – S-phase

Introduction

In experimental and clinical oncology proliferative activity is one of the most interesting cell biological parameters. The identification of DNA synthesizing cells has mainly been restricted to autoradiography [8, 14]. In contrast to 3H-thymidine the newly established BrdUrd technique [4, 12] allows the estimation of the S-phase cell frequency within several hours and offers the advantage of simple and safe handling.

It has been shown in cell cultures and in experimental tumour cells that the BrdUrd data correspond to the 3H-thymidine results [9] in cycling cells.

Gunduz [5], Hoshino et al. [7] and Wilson et al. [19] determined the proliferating fraction of cell populations of different solid human tumours by the BrdUrd labelling technique. They obtained BrdUrd labelling indices which correlate closely to the 3H-thymidine labelling indices or to the FCM data on small number of cases. In the present study we have applied the BrdUrd technique to 27 human renal cell carcinoma. In parallel FCM measurements and 3H-thymidine labelling of the same tumour have been performed.

Material and Methods

Materials

The tissue samples were won during tumour nephrectomy, stored for transportation in 0.9% NaCl solution and labelled within 1–2 h.

Cell Dispersion

The samples were rinsed in F10 medium (Boehringer), cut with scalpels in fresh medium and stirred for 5 min at room temperature. The F10 medium was chosen because of its low phenol red concentration (1.2 g/l) compared to medium 199 (15.6 g/l). Phenol red is said to influence cell kinetics. Afterwards, the suspension was filtered through a nylon mesh (pore diameter 50 μm). In order to obtain further dispersion, the remaining tissue particles were stirred in 0.5% pepsin-HCl solution (Serva) for 15 min. At intervals of 5 min, small amounts (1 ml) of the suspension were taken out for staining and following FCM measurement.

Fluorescence Measurement

For flow cytometry, the mechanically separated as well as the pepsin treated cells were stained with ethidium bromide/mithramycin following Zante et al. [20]. DNA fluorescence measurements were performed using a mercury arc lamp based FCM (Partec, Switzerland). The evaluation of S-phase cell percentages of the tissues from flow cytometry histograms was performed as proposed by Göhde et al. [3] and Meier [11].

Some of the normal kidney cell suspensions did not produce acceptable histograms, because of problems in cell dispersion.

FCM measurement was performed after different staining times, from 1–24 h in order to optimize the results for the individual samples.

BrdUrd Monoclonal Antibody Labelling

The mechanically dispersed cells suspended in F10 medium were incubated for 1/2 to 1 h in medium containing 15 μM bromodeoxyuridine-deoxycytidine solution (equimolar amounts; Serva). After

Table 1. Renal cell carcinoma. Tumour samples with one cell line

No.	Age/Sex	Staging and Grading[a]		a.e.[b]	%S-phase cells (FCM)	LI (%) BrdUrd	LI (%) 3H-TDN
1	47/m	$T_1N_1M_0(G_1)$		–	4.1	1.9	–
2	53/f	$T_2N_0M_0(G_2)$		–	5.6	52.0	–
3	73/f	$T_2N_0M_0(G_2)$		–	4.3	21.4	–
4	60/m	$T_2N_0M_0(G_2)$		–	7.8	17.8	–
5	66/m	$T_1N_0M_0(G_1)$		–	2.4	5.4	5.7
6	72/f	$T_3N_1M_0(G_2)$		+	3.2	3.0	1.6
7	49/f	$T_2N_0M_0(G_1)$		–	1.5	6.9	–
8	55/f	$T_3N_0M_1(G_2)$		–	7.2	2.8	–
9	58/m	$T_3N_1M_0(G_3)$		+	7.5	6.0	–
10	44/m	$T_2N_0M_0(G_2)$		–	13.6	19.9	–
11	54/m	$T_2N_0M_0(G_2)$		–	3.5	0.5	–
12	40/m	$T_3N_0M_0(G_1)$		–	4.7	0.8	–
13 a)	71/f	$T_2N_0M_0(G_1)$		+	6.5	14.6	–
b)		normal kidney			6.0	2.9	–
14 a)	66/f	$T_2N_0M_0(G_1)$		–	6.5	7.9	0.8
b)		normal kidney			3.4	0	0.1
15 a)	49/m	$T_3N_0M_0(G_2)$		+	[c]	2.3	0
b)		normal kidney			[c]	0.5	0
16 a)	59/m	$T_1N_0M_1(G_2)$		–	1.3	3.1	–
b)		normal kidney			2.4	2.4	–
17 a)	57/f	$T_2N_0M_0(G_2)$		+	1.5	7.4	–
b)		normal kidney			[c]	3.4	–
18 a)	50/m	$T_3N_1M_1(G_3)$	tumour wall	–	3.1	13.3	–
b)			tumour center		14.5	2.0	–
19 a)	80/f	$T_3N_0M_0(G_2)$	tumour wall	–	6.1	6.7	–
b)			tumour center		5.7	1.2	–

[a] Staging see [16], grading see [6]
[b] a.e.: after embolisation
[c] The calculation of S-phase frequency of these histograms was not possible because of insufficient DNA-histograms

incubation the cells were fixed in 70% ethanol. For antibody labelling the cells were removed from the ethanol and resuspended in 1.5 n HCl for 20 min at room temperature, washed twice in phosphate-buffered saline (PBS) and incubated in PBS containing 0.5% TWEEN 20, 0.5% bovine serum albumin (BSA) and a 1:500 dilution of monoclonal anti-BrdUrd (Partec, Switzerland) for 1 h at 37 °C. After this treatment the cells were washed once in PBS and incubated in PBS containing 0.5% TWEEN 20, 0.5% BSA and 1% fluorescein labelled goat anti-mouse IgG. After 10 min to several h the S-phase cells show a bright fluorescence when stimulated. The labelling index was determined by counting the flourescent cells [9].

3H-Thymidine Labelling of DNA

Cell suspensions were incubated in F10 medium containing tritiated thymidine (1 μCi/ml; 37 KBq/ml, spec. activity 925 GBq/ml; Amersham) for 15 min at 37 °C and fixed in 70% ethanol. The cells were mounted on slides and covered with photographic emulsion (Ilford, K5). After exposure for two days at 4 °C in the dark, the slides were developed (Kodak, D9). The labelling index was calculated.

Results

The results of the tests are shown in Tables 1 and 2. The normal kidney tissue samples (nos. 13b, 14b, 15b, 16b, 17b, 24b and 26b), measured as reference, show S-phase cell frequencies of about 3%, the labelling indices (LI) range from 2.5 to 4%. Sample no. 25b is out of range. This sample with an LI of about 10.3% possibly was taken from the tumour periphery.

In tumour tissue samples generally, we observed a high variability of LI values which range from 1.0 to 20% (Tables 1 and 2).

According to their labelling index the tumours with only one cell line, presumable "diploid samples", can be divided into three groups: one group with an LI of about 1.5 to 3% similar to normal kidney tissue and two other groups presenting LI above 3% up to 8% and above 8% up to 20%. Sample no. 2 – LI of 52% – cannot be integrated into this scheme.

The S-phase values calculated from flow cytometry histogram analysis varied from 1.5 to 14.5%. Only in a few cases did the LI and the FCM data have the same order of magnitude (nos. 6, 9 and 19a).

The tumours with one cell line showed an interrelationship between status of cell differentiation [12, 13] and proliferative activity as measured by flow cytometry (Table 3). Well differentiated tumours showed a relatively low S-phase cell frequency; the S-phase cell frequency increased with decreasing cell differentiation.

Table 2. Renal cell carcinoma. Tumour samples with one or more anueploid cell lines

No.	Age/Sex	Staging and Grading[a]		a.e.[b]	%S-phase cells (FCM)	LI (%) BrdUrd	LI (%) 3H-TDN
20	42/m	$T_2N_0M_0(G_2)$		–	[d]	13.7	–
21	50/m	$T_3N_1M_1(G_3)$		–	[d]	1.0	–
22	76/m	$T_2N_0M_0(G_2)$		–	4.5	3.9	–
23	57/m	$T_1N_0M_1(G_3)$		–	[d]	3.2	–
24 a)	58/m	$T_2N_0M_1(G_2)$		+	4.6	16.7	1.5
b)		normal kidney			[c]	3.4	0.9
25 a)	52/m	$T_3N_0M_0(G_2)$		–	4.1	0.5	–
b)		normal kidney			3.3	10.3	–
26 a)	65/f	$T_3N_0M_0(G_2)$		+	4.2	2.2	–
b)		normal kidney			3.3	4.2	–
27 a)	40/m	$T_2N_0M_0(G_2)$	tumour wall	–	8.3	2.5	–
b)			tumour center		6.3	0.6	–

[a] Staging see [16], grading see [6]
[b] a.e.: after embolisation
[c] The calculation of S-phase cell frequency of these histograms was not possible because of insufficient DNA-histograms
[d] The calculation of S-phase frequency of these multiclonal tumour probes was not possible because of overlapping of cells in different ploidy stages

Table 3. Interrelationship of Grading, TNM-System, %S-phase Frequency and LI after BrdUrd Labelling

	Tumours with one cell line		Tumours with aneuploid cell line(s)	
	%S-phase cells (FCM)	LI (%) BrdUrd	%S-phase cells (FCM)	LI (%) BrdUrd
Grading				
G_1	4.3 (6)	6.3 (6)	–	–
G_2	5.4 (11)	11.5 (12)	5.3 (6)	5.7 (7)
G_3	8.4 (3)	7.1 (3)	–	2.1 (2)
TNM				
T_1	2.6 (3)	3.5 (3)	–	–
T_2	5.6 (9)	16.5 (9)	5.9 (4)	7.5 (5)
T_3	6.5 (8)	4.2 (9)	4.2 (2)	1.2 (3)
N_0	5.7 (16)	9.7 (17)	5.3 (6)	5.4 (8)
N_+	4.8 (4)	7.9 (5)	–	–
M_0	5.3 (16)	11.0 (17)	5.5 (5)	3.9 (6)
M_+	6.5 (4)	5.3 (4)	4.6 (1)	10.0 (3)

Number of tumour samples in brackets

Compared to the FCM data the LI was slightly increased in highly differentiated tumours. The value of LI doubled in tumours with moderately differentiated cells, but decreased beneath the value of flow cytometry data in low differentiated tumours.

The increase of S-phase cell percentage and increase and decrease of LI is obviously dependent on tumour size in the same way (Table 3). T_2 tumours showed an extremely high LI value, as we observed in G_2 tumours.

Tumours without metastasis (M_0) or lymphnode infiltration (N_0) were moderately differentiated. Their LI value was high.

The tumours with one or more aneuploid cell lines (Table 2) were divided into two main groups according to BrdUrd labelling. The low proliferating group had an LI below 4%, similar to the LI of normal kidney tissue samples, and the high proliferating group had an LI up to 17%.

In most cases flow cytometry histogram analysis was not possible because of the overlapping curves of the different tumour cell lines. A further aspect of our results was the dependance of S-phase cell frequencies on the tumour location the sample has been taken from. Samples no. 18a, 19a, and 27a taken from the tumour periphery, had a higher labelling index, between 2.5 to 13.3%, in comparison to the samples taken from the centre of the same tumour. Samples no. 18b, 19b, 20 and 27b show values of 0.5 to 2% even below of the LI of normal kidney tissue.

The close correlation between 3H-thymidine and BrdUrd labelling indices we described in experimental tumours [9] has not been reproduced here due to the fact that kidney tumours did obviously not accept 3H-thymidine in a comparable manner.

Discussion

In previous BrdUrd studies we showed, that the technique can be used to measure the labelling index of proliferating experimental cells [9]. In this study the same technique has been applied to clinical material. In contrast to the experience gained from experimental tumour cells human kidney tumour was significantly different in BrdUrd labelling indices and in the 3H-thymidine labelling indices (Tables 1 and 2).

Tumours of different histological classification showed extremely low 3H-thymidine LI whereas BrdUrd labelling results correlated with the expected results. There was only one exception of a highly differentiated tumour sample (no. 5) where both labellings gave identical results. Earlier publications [13, 2] already mentioned the reduced 3H-thymidine acceptance of solid tumours. Frankfurt [2] suggests that endogeneous thymidine via de novo synthesis is preferred in comparison to the exogeneous 3H-thymidine by salvage in DNA synthesis. Other authors [1, 10, 15, 17, 18] report comparatively high 3H-thymidine LI in solid tumours. These authors have not worked with single cell suspensions but with tissue sections. In solid tissue the acceptance of exogeneous and endogeneous substances is obviously not comparable with the conditions in a cell suspension.

Wilson et al. [19], obtained good correlation of the results with the two labelling techniques after incubating fragments of different solid human tumours in 3H-thymidine and BrdUrd containing medium respectively. However they performed 3H-thymidine incubation using a high pressure of oxygen as described by Steel and Bensted [13].

Gunduz [5] incubated single cell suspensions of human breast cancer samples. In contrast to our observations he found a close correlation between the labelling indices after BrdUrd and 3H-thymidine incubation.

Tables 1 and 2 show, that the FCM derived S-phase frequencies can be lower than the BrdUrd LI. This difference might represent a cell fraction not actually in the S-phase at the time of resection but which is capable of immediate initiation of DNA synthesis in the growth conditions provided by an optimal medium like F10 medium. On the other hand, Hoshino et al. [7], who adminstered BrdUrd to brain tumour patients and estimated the BrdUrd LI of biopsy specimen of these patients as well, found that the LI of in vivo and in vitro samples agreed, with the flow cytometry data.

It seems possible that intercellular adhesion and thus the effective resorption surface for DNA precursor uptake play an important role in different tumour types.

In contrast to these findings our data about peripheral and central parts of tumours suggested that tumours contain different proportions of resting S-phase cells. This could be shown on three kidney tumours nos. 18, 19 and 27 (Tables 1 and 2). The presence of resting S-phase cells was supported by our data taken from experimental cell lines. After the end of logarithmic growth the discrepancy of LI values and FCM data increased [9]. Generally, we observed a high variability of S-phase values even within the same patient tumour. This supported the view that the exact location of tumour biopsy predicted the results. It is well known that vascularisation, influences the size of the proliferating compartment of the tumour cells. Therefore, we took the opportunity to analyse samples from different regions of the same tumour. The equal or even higher FCM determined S-phase cell frequencies in the center of tumours can be explained by the presence of resting S-phase cells also in human tumours (Tables 1 and 2).

Our data support the value of the BrdUrd labelling technique in studying proliferating activity of human tumours, because: BrdUrd was better incorporated in the proliferating cells than 3H-thymidine at least in our study on suspended cells. Comparing different labelling techniques the procedure delivering the highest S-phase cell frequencies has a higher degree of accuracy. Therefore the BrdUrd method seems to be the more suitable method to estimate S-phase cells in solid tumours. The BrdUrd data on human tumours were closer to the FCM results. The flow cytometry method measured only the DNA distribution of cells and was not able to discriminate between DNA synthesizing and resting S-phase cells. The BrdUrd labelling method only detected proliferating cells but gave no information about the variety of stemlines and DNA indices.

The application of both methods, FCM and BrdUrd labelling technique in parallel allows investigation of the proliferative characteristics of spontaneous tumours. Such data can be derived from clinical specimens and might be important in the determination of appropriate chemotherapy.

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