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The Kinetics of Cell Proliferation in Wilms' Tumours

Investigations with an Autoradiographic *In Vitro* Method

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Abstract—*The proliferation kinetics of* 11 *Wilms' tumours* (9 *primary tumours*, 2 *lung metastases*) *were studied with an autoradiographic* in vitro *method, simple labelling with* 3H-*thymidine and double labelling with* 3H- *and* 14C-*thymidine. The results were in accordance with the clinical experience of rapid tumour growth. The* 3H-*thymidine labelling index ranges between* 22.4 *and* 46.3%, *the mean cell cycle time between* 11.2 *and* 22.1 hr, *the DNA synthesis time between* 8.5 *and* 13.8 hr, *and the mitosis time between* 0.3 *and* 1.5 hr. *The growth fraction, which can be determined only approximately with* in vitro *methods, showed an average value of* 0.5. *The growth of* 2 *lung metastases is not different from the pattern of proliferation of other primary Wilms' tumours.*

The proliferative activity of Wilms' tumours reaches the magnitude of rapidly proliferating experimental animal tumours.

Since X-rays and most cytostatics show specific activity in dependence on phase of the cell cycle or the proliferative behaviour, the cytokinetic data of individual tumours allow the formulation of an index, which represents a general measure for the sensitivity of tumour cell to chemotherapy and radiation. For Wilms' tumours this "Cytokinetic Therapy Index" ranges between 0.62 *and near* 1. *This is in a region of high sensitivity. The fundamental importance of proliferation kinetics for the treatment of malignant individual solid tumours in children is discussed.*

INTRODUCTION

WILMS' tumours are dysontogenetic tumours with high degree of malignancy. Genesis and particular biological behaviour delimitate these tumours from the typical malignomas of adult age. Accordingly, particularities may be expected of the pattern in cell proliferation of clinical relevancy.

Malaise *et al.* [1] have calculated the growth rates of 242 tumours and compared them with the histologic type. Their calculations were based on a review of the literature and on their own results. Five Wilms' tumours and 1 embryonal carcinoma were to be ascribed to the tumours with the most rapid growth. This calculation, however, is based only on the labelling index (*LI*). In contrast, the double labelling method according to Pilgrim and Maurer [2] allows *in vitro* the precise determination of the DNA synthesis time and of the dependent cell cycle parameters (mean generation time, mitosis time, duration of the G- and G1-phases). The results are comparable to those of the PLM method [3].

The results of the investigation on cell kinetics of 11 Wilms' tumours are reported.

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MATERIALS AND METHODS

Immediately after the extirpation small pieces without necroses were taken from a marginal region of 11 Wilms' tumours (9 primary tumours, 2 lung metastases). The particles were put into cooled Hanks' solution (4°C) and transported to the laboratory. The tumour pieces were cut into small particles of about 1–3 mm^3 by means of star-scalpels. Three specimens, each containing 2–3 tumour particles, were incubated at 37.5°C with normal air P_{O_2}. Each incubation tube contained

1.6 ml buffered Parker's medium, 0.4 ml 10% calf serum, and 1 μCi/ml 3H-thymidine (spec.act. 11.8 Ci/mmole). Incubation was stopped by addition of 4% neutral formalin after 120 min (cases 1/74, 4/74, 7/74 and 17/74 after 60 min).

The calculation of the DNA synthesis time was performed with the method of double labelling [2] using 3H- and 14C-thymidine: 3 specimens with 2–3 tumour particles each were incubated with 1 μCi/ml 3H-thymidine (spec.act. 25.3 Ci/mmole) over 60 min. The incorporation of 3H-thymidine was stopped by rinsing in ice-cooled Hanks' solution, and then the incubation was continued in fresh medium containing 1 μCi/ml 14C-thymidine (spec.act. 53 mCi/mmole) for another 60 min. Then the material underwent formalin fixation and embedding into paraffin. Three to four micrometer thin sections were prepared, paraffin was carefully removed and covered with K6-fotoemulsion (VEB ORWO, WOLFEN/GDR), shortly dried with air and then exposed in dark boxes at 4°C for about 18 days. The double labelled autoradiograms were covered with one single thick layer of K6-fotoemulsion and exposed for 12 days. The photographic development was performed with ORWO developer M-H-28 and fixed with ORWO fixation salt A300. Then, the autoradiograms were stained with hematoxylin and covered in glycerolgelatine.

The evaluation of autoradiograms was carried out by counting an average of 6000 cells from samples with simple labelling and 400–600 labelled cells resp. with double labelling. The incubation with normal air P_{O_2} led to a labelling only of marginal zones (see [4]). We avoided errors in calculation by measuring the marginal zone with an ocular-measuring-device (VEB Carl Zeiss, Jena/GDR) and by restriction on to a 100 μm large zone or by evaluation of marginal tumour particles.

Calculation of cell cycle parameters

Mendelsohn [5, 6], Steel [7, 8] and Lennartz *et al.* [9] described in detail the possibilities and error sources of autoradiographic methods.

1. 3H-*thymidine-labelling-index* (*LI*). The *LI* is the expression of the percentual proportion of 3H-labelled cells, i.e., the cells in DNA synthesis, to the total number of cells at a certain time:

$$LI = \frac{N_S}{N} \ (\%).$$

The *LI* is higher with the higher proliferative activity of a cell population. Thus, the *LI* is an informative parameter for the characterization of cell proliferation.

2. *Duration of the DNA synthesis phase* (Ts). This parameter is calculated with the double labelling technique according to Pilgrim and Maurer [2]. The numeric ratio between the cell nuclei with mixed 3H/14C-labelling and those with isolated 3H-labelling is in direct correlation with the amount of time shifting between the two labelling processes [10]. Cell nuclei with 3H- and 14C-labelling may be differentiated exactly on the basis of their different radiation energy and nuclei traces, respectively. Thus

$$\frac{\text{duration of S-phase}}{\text{time interval d}t} = \frac{\text{No. of 3H/14C-labelled nuclei}}{\text{No. of isolated 3H-labelling}}$$

and

$$Ts = \frac{N\,3\text{H}/14\text{C}}{N\,3\text{H}} \times \mathrm{d}t.$$

3. *Mean cell cycle time* (Tc). It is defined by the ratio of DNA synthesis time to number of cells in *S* to the total cell number, which is expressed in the *LI*. Since this form of the relationship is true only for steady-state growth, it must be corrected by the factor λ if tumours are concerned [8]. λ In human tumours amounts to 0.7–0.9; in general it is near 0.75.

$$Tc = \frac{Ts}{LI} \times \lambda.$$

With very short duration of cell cycle the value of the factor λ is near 0.9. Therefore, the last value must be used for the calculation of *Tc* in cases 4/74 and 4/76.

4. *Mitosis time* (Tm). We calculated *Tm* according to Reiskin and Mendelsohn [11] from the following relation: the mitosis time to the DNA synthesis time are proportional to the number of cells in mitosis (expressed in the mitosis index) to the number of cells in *S*, which is obtained in *LI*:

$$Tm = \frac{Ts \times MI}{LI}.$$

The mitosis index (*MI*) was determined on sections with Feulgen staining of the original tumours.

5. *Postmitotic G1- and premitotic G2-phase* (T $G1+G2$). The duration of the G2-phase cannot be calculated with the method we used. The time TG2 can be determined *in vivo* only. Therefore, the duration of G1- and G2 phases is obtained from

$$T\ (G1+G2)=Tc-(Ts+Tm).$$

In general, a G2 phase in Wilms' tumours can be expected to take 0.5–2.0 hr, so that the duration of the G1 phase can be calculated.

6. *Growth fraction* (GF). The calculation of *GF* is still difficult in human tumours using *in vitro* methods. An approximation is possible only in experimental tumours [6, 12]. The growth fraction expresses the number of proliferating cells to the total number of cells. In order to obtain the *GF* also for human tumours Muggia *et al.* [13] assume that this value is represented by the actually determined 3H-thymidine-labelling-index (*LI* actual) to the ratio of number of cells in *S* to the total number of proliferating cells (*LI* theoretical):

$$GF=\frac{LI\ \text{act.}}{LI\ \text{th.}} \text{ and } GF=\frac{LI}{0.8\times\frac{Ts}{Tc}}.$$

7. *Potential tumour doubling time* (Td). The *Td* is clinically important. It is formed from the duration of the cell cycle and the number of proliferating cells in the tumour, which are registered in the *GF*. Since enlargement of the tumour does not only mean increase of the absolute number of proliferating cells but also an increase of non-proliferating cells, the logarithmic relation exists [9, 14]:

$$\frac{\log 2}{Td}=\frac{\log\ (1+GF)}{Tc} \text{ and}$$

$$Td=\frac{\log 2}{\log\ (1+GF)}\times Tc.$$

The potential tumour doubling time does not include the amount of cell loss.

8. *Cytokinetic therapy index* (CTI). The close relationship between kinetics of cellular proliferation in tumours and therapeutic efficiency may be summarized in an index, which is called "Cytokinetic therapy index". This term includes the time relation of usually sensitive phases of cell cycle (*S*, G2, and *M*) to the total duration of the cell cycle:

$$CTI=\frac{T\ (S+G2+M)}{Tc}.$$

If a cytostatic agent has an *S* phase specifity, then

$$CTIs=\frac{Ts}{Tc}.$$

This way, the probability of the cell kill quote during the chemotherapy can be estimated. The more CTI is nearing to the value 1, the more a lethal effect may be expected from the cytokinetic view-point with the application of phase-specific cytotoxic agents.

As we cannot calculate the G2 phase duration, we assumed a maximal value of 2 hr according to the duration of cell cycles of experimental tumours and of rapidly growing tumours in men. In Wilms' tumours this value is partly lower.

9. *Cell loss* (*CL*) The amount of cell loss cannot be calculated by the method used.

RESULTS

1. The autoradiographic *in vitro* method of 3H-thymidine labelling of tumour cells and the method of double labelling with 3H- and 14C-thymidine are suitable for the determination of parameters of proliferation kinetics in solid tumours with a standardized examination procedure. 3H-thymidine labelling index, DNA synthesis time, mean cell cycle time, growth fraction and potential tumour doubling time (without cell loss) can be determined.

2. Wilms' tumours belong to the group of tumours with extraordinarily rapid proliferation (Table 1). Their growth rate is like that of many experimental tumours. The *LI* ranges between 22.4 and 46.3% (average 33.2%), the mean cell cycle time is always below 24 hr (11.2–22.1, average 15.1 hr), and the DNA synthesis times are short (8.5–13.8, average 10.6 hr). The mitosis times are similarly short (0.3–1.5 hr). Together with growth fraction, the potential tumour doubling times (without cell loss) range from 15.1 to 34.5 hr (average 23.3 hr). The growth fraction ranges from 0.45 to 0.67. Although the calculation of the *GF* is based on theoretical considerations, it comes near to the actual conditions. This has been demonstrated by studies on other types of tumours with different methods (see [15, 16]).

3. We examined the proliferation kinetics of lung metastases of 2 cases. There was no difference as compared to other primary Wilms' tumours. The *LI*s of metastases are 45.2 and 32.7%, and the cell cycle times

Table 1. *Data of the proliferation kinetics of Wilms' tumours*

No.	*LI* (%)	*Tc*	*Ts*	*Tm*	*T*(G1+G2)	*GF*	*Td*	*MI* (%)
1/74	22.7	19.8	10.0	1.5	8.3	0.56	30.9	3.4
7/74	29.8	15.7	10.5	0.7	4.5	0.56	24.0	2.1
17/74	22.4	22.1	11.0	0.8	10.3	0.56	34.5	1.6
2/75	30.8	11.8	9.8	0.6	1.4	0.46	21.6	1.95
4/75	37.4	12.0	10.0	0.3	2.9	0.56	18.7	1.6
5/76	25.3	15.0	8.5	0.6	5.9	0.56	23.4	1.7
14/76	46.3	11.2	9.6	0.3	1.3	0.67	15.1	1.43
20/76	37.3	16.6	13.8	0.7	2.1	0.56	25.9	1.9
22/76	35.2	12.2	9.5	0.5	2.2	0.56	19.0	1.7
Metastases								
4/74	45.2	11.7	9.8	0.3	1.6	0.67	15.8	1.6
16/76	32.7	17.6	12.8	0.7	4.1	0.55	27.9	2.55

Cell cycle times in hr. *Ts* estimated 10 hr each for cases 1/74 and 4/75.

are short with 11.7 and 17.6 hr, respectively. Also the DNA synthesis times (9.8 and 12.8 hr), the mitosis times (0.3 and 0.7), and the potential tumour doubling times (15.8 and 27.9 hr) did not deviate from the usual pattern. Only the high *GF* of 0.67 in case 4/74 is uncommon.

4. The calculated growth rate is in full accordance with the clinical observations of a short-term clinical symptomatology, particularly, if the extraordinary volume of Wilms' tumours at the time of diagnosis is taken into consideration (tumour weight ~ 0.5–1 kg).

5. The "cytokinetic therapy index" ranges between 0.62 and about 1 (Table 2) with an average of 0.89. If only the DNA synthesis time is used for calculation (*CTIs*), the values obtained are still between 0.50 and 0.86 (average 0.72). Like the primary tumours, the *CTI* of lung metastases is near the ideal value with figures of 0.88 and 0.95. Thus, the *CTI* is high in each case. This is in full accordance with the extraordinary sensitivity of Wilms' tumours against cytostatics and irradiation.

Table 2. *The "cytokinetic therapy index" of Wilms' tumours*

No.	*CTI*	*CTIs*
7/74	0.84	0.66
17/74	0.62	0.50
2/75	~1	0.83
5/76	0.74	0.57
14/76	~1	0.86
20/76	0.99	0.83
22/76	0.98	0.78
Metastases		
4/74	0.95	0.84
16/76	0.88	0.73

CTI—related to $T(S+G2+M)$; *CTIs*—related to *Ts*.

6. The DNA synthesis times were assumed to be 10.0 hr for the cases 1/74 and 4/74 according to the average of the calculated *Ts* of the other cases. These DNA synthesis times may be regarded as rather real. They are the basis for the calculation of depending parameters of the cell cycle.

DISCUSSION

The examination of the proliferation kinetics of human tumours for the characterization of growth behaviour is well possible with autoradiographic *in vitro* technique. The *in vitro* results are representative for *in vivo* conditions with strict consideration of a time factor. This has been demonstrated by comparative *in vivo–in vitro* and *in vitro–in vivo* studies [4, 17, 18]. The used *in vitro* method is highly efficient also in comparison with the method of labelled mitosis (*PLM*), which can be used only *in vivo* [3, 9, 19]. The application of tritium-labelled thymidine *in vivo* cannot be used in children, because of the risk of genetic lesions due to radiation [20, 21].

The data on proliferation kinetics of 9 primary Wilms' tumours and 2 lung metastases showed extraordinary rapid growth. The growth rate reaches the magnitude of various rapidly proliferating experimental animal tumours [14, 17, 22–24]. The mean cell cycle times remain uniformly below 24 hr and the DNA synthesis times and mitosis times are short. The growth fraction is about 0.5. Malaise *et al.* [1] obtained similar results. They calculated an average mean duration of cell cycles of 1.66 days after determination of the *LI* and an estimated DNA synthesis time of 16 hr in 5 Wilms' tumours.

In general, the metastases are said to grow

more rapidly than their primary tumours. This is concluded by several authors [25–27] from measurements of doubling times of lung metastases on thorax X-ray pictures in comparison with primary tumours. The finding of more rapid growth of metastases is also confirmed with the experimental Lewis lung carcinoma [24].

The growth of lung metastases of our 2 cases, however, is not different from the growth of other primary Wilms' tumours. This may be due to the usually very high proliferative activity of Wilms' tumours, which is reached only in single cases of different types of solid tumours in children [28]. Therefore, differences in the growth behaviour of the primary Wilms' tumours and its metastases are not evident or they are absent. This statement seems justified, although data on proliferation kinetics of these primary tumours are not available in these 2 cases.

The extraordinary growth potential of Wilms' tumours is in accordance with the clinical observations of short tumour histories, high weight of the tumour at the time of diagnosis, and the usually rapid increase in volume in relapses or metastases. Carcinoma of adult patients, however, almost exclusively grow more slowly, the variability of the cell cycle time is essentially defined by long G1 phases [29–31]. The tumour individuality is more pronounced in other solid tumours in children than in Wilms' tumours, where it is less apparent due to the always high proliferation rate [32].

Examinations of proliferation kinetics of malignant tumours in men are of outstanding practical interest. The effect of cytostatics and X-irradiation depends directly, although not exclusively, on the proportion of proliferating cells in the tumour, on the mean duration of the cell cycle and the relative length of its partial phase [18, 33–35]. Therefore, a general prediction is possible on the effectiveness of chemotherapy on the basis of cytokinetic data of individual tumours. The relations may be expressed by the "cytokinetic therapy index". This *CTI* is usually high in Wilms' tumours. The clinical experience with the sensitivity of Wilms' tumours to chemotherapy and irradiation confirms this fact.

Today, the treatment of solid tumours in childhood follows a well-established pattern in many countries. The results obtained this way are splendid, particularly those of Wilms' tumours. The 5 yr survival rate is about 70% [36]. Considering the knowledge of basic data of proliferation kinetics and sensitivity of tumours to cytostatic agents it seems possible to adjust therapy to the individual peculiarities and thus render it more adequate. This opens new possibilities for improved therapeutic results.

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