X-irradiation effects on thymidine kinase (TK):

I. TK1 and 2 in normal and malignant cells

Q. He*, S. Skog*, I. Welander† and B. Tribukait†

*Department of Oncology, KFC, Huddinge University Hospital AB, S-141 86, Stockholm, Sweden and

†Department of Oncology and Pathology, Section for Medical Radiobiology, CCK, Karolinska Institute, S-171 76 Stockholm, Sweden

Received 5 July 2001; revision accepted 21 July 2001

Abstract. The effect of radiation on TK is more complicated than would be expected from earlier results on bone marrow cells (Feinendegen *et al.* 1984, *Int. J. Radiat. Biol.* **45**, 205). TK activity increased at 0.01 Gy and then decreased up to 1 Gy in mouse spleen. In contrast to the results for the spleen, an increase in activity at 0.1 Gy was seen in mouse thymus. The activity of dephosphorylated TK1 (TK1a) in both spleen and thymus was reduced to 50% after irradiation at 0.5–1 Gy. The degree of phosphorylation (TK1b/TK1a ratio) changed in spleen, but not in thymus. The activity of TK2 in mouse liver increased at 3 h after 5 Gy by about 60%. In mouse ascites tumour, a dose-independent (1–5 Gy) oscillating TK1 activity was found up to 24 h, especially for TK1a and TK1b. The amount of TK1 was unchanged up to 12 h, but decreased at 24 h. This suggests that the differences in the changes in the degree of phosphorylation of TK1 after irradiation among spleen, thymus and ascites tumour further underline the complexity of the response of TK1 activity to irradiation. The dramatic change in the activities of TK1a and TK1b may illustrate that both of them are more radiosensitive than TK-h, a variant with mixed TK1 and TK2 properties.

INTRODUCTION

Thymidine kinase (TK) is one of the key enzymes in DNA metabolism, catalysing the phosphorylation of thymidine to thymidine monophosphate (Feinendegen *et al.* 1984; Arnér & Eriksson 1995). In X-irradiation studies on TK of mouse bone marrow cells, Feinendegen and co-workers (Feinendegen *et al.* 1984) found an extremely high sensitivity of TK activity to ionizing radiation. Whole-body exposure to gamma-irradiation as low as 0.01 Gy resulted in temporary and partial inhibition of TK activity. The maximum inhibition was seen 4 h after irradiation and the effect disappeared 6 h later. Split-dose irradiation with intervals of 30 min and 4 h resulted in a more rapid and almost immediate recovery of TK activity and could indicate the existence of both inhibitory and stimulatory effects of ionizing irradiation on TK activity. Certain concentrations of NaHCO₃ and pH values in the medium of the cell extract were necessary to maintain the inhibition of TK activity, indicating the need for specific environmental conditions. Because procain

chloride prevented the irradiation-induced inhibition of TK activity, and as this compound changes the membrane fluidity, involvement of cell membrane was suggested (Hohn-Elkarim *et al.* 1990). Manipulation of the cellular glutathione content also changed the expression of TK activity. Therefore, Hohn-Elkarim and co-workers (Hohn-Elkarim *et al.* 1990) suggested that SH-containing compounds were able to control TK activity.

The present study is part of a series of investigations of deoxynucleotide metabolism in irradiated cells. Here we investigated the TK activity of mouse spleen, thymus, liver and ascites tumour, representing cell systems of various degrees of proliferation activity and radiosensitivity. The cell systems also differ considerably in total TK activity and the proportion of various TK1s. The differential behaviour of the variants of TK1 in cytoplasm and TK2 in mitochondria does not seem to have been studied. From previous studies in ascites tumour cells it is known that the half-life of TK activity is about 45 min (He *et al.* 1991). The irradiation-induced inhibition of TK activity could, thus, be due to inhibition of the synthesis of TK1, resulting in a lower cellular concentration of TK1. In the present study we therefore examined, in addition to TK1 activity, the cellular concentration of TK1 after X-irradiation. The amount of TK1 was determined by Western immunoblotting using an affinity-purified anti-TK1 polyclonal antibody (pAb1), developed in our laboratory (He *et al.* 1996b). In order to exclude the possibility of different reactions of the variants of TK1, those TK1s were also isolated by isoelectric focusing and their activity was measured separately. We confirmed the significance of well-controlled pH and NaHCO₃ conditions for the reproducibility of TK measurements as found by Feinendegen *et al.* (1984).

MATERIALS AND METHODS

Cell materials

Cells from spleen, thymus and liver were prepared from 3-month-old female Naval Medical Research Institute (NMRI) mice. The organs were removed from the animals and squeezed in a Tris-buffer (100 mM Tris, 70 mM NaCl and 5 mM EDTA, pH 7.5) gently through nylon net (40–50 mesh count/cm) to obtain a single-cell suspension. Ehrlich ascites tumour (EAT) cells were passaged by the injection of 2×10^7 cells i.p. into 3-month-old female NMRI mice every 10th day.

Cell culture

For *in vitro* irradiation experiments, EAT cells growing *in vivo* were removed from the animals 4 days after transplantation, washed in Tris buffer (Tris 100 mM, NaCl 70 mM and EDTA 5 mM, pH 7.5) and then resuspended in RPMI medium, supplemented with 10% calf serum, L-glutamine (1×10^{-3} M) and PEST [penicillin 2.4 IU and streptomycin 2.4 µg per ml, respectively (Flow lab, Life Technology, Gothenburg, Sweden)] to a final concentration of 5×10^6 cells/ml. The cells were incubated at 37 °C and counted microscopically in a haemocytometer, and cell viability was tested by dye exclusion.

Irradiation

The mice were whole-body roentgen irradiated at 250 kV, 15 mA and 0.5 mm Cu added filtration, SSD 50 cm, at a dose rate of 1.33 Gy/min for doses of 0.1–5 Gy. For irradiation with doses of 0.01 and 0.05 Gy a dose rate of 0.67 Gy/min was used (250 kV, 3 mA and 0.5 mm Cu filtration, SSD 50 cm). The animals moved freely in well-ventilated perspex boxes. The dosimetry was based

on lithium fluoride dosimetry and was controlled routinely by a Simplex Universal Dosimeter (Kebo Lab AB, Stockholm, Sweden).

Cells growing *in vitro* were irradiated at 37 °C, 20–24 h after suspending the cells in medium, when they were growing exponentially.

Determination of the cell cycle composition

The distribution of the cells in the cell cycle was determined using flow-cytometry as reported earlier (Tribukait *et al.* 1975). The cells were fixed in ice-cold ethanol. Cell nuclei were stained with ethidium bromide after pepsin and RNase treatment and analysed using a flow-cytofluorometer (ICP 11, Phywe, Münster, Germany) with a flow rate of up to 1000 cells/s. The relative numbers of G₁, S-phase and G₂ cells were calculated from the areas of the DNA histogram assuming a Gaussian distribution of the G₁ and G₂ maximum and attributing the remaining part to the S-phase. The proportion of S-phase cells calculated was in good agreement with the [³H]-dTdR labelling index (Skog & Tribukait 1985).

Determination of TK activity

Cells of spleen, thymus and liver and the EAT cells were suspended in cold Tris-buffer (Tris 100 mM and NaCl 70 mM, pH 7.5) and washed twice. The cells were then prepared in a lysing Tris-buffer (Tris-HCl 10 mM, MgCl₂ 5 mM and NaF 5 mM, pH 7.0) containing 1300 mg/l NaHCO₃ and 2 mM β-mercaptoethanol and sonicated for 20 s at 4 °C (Cell Sonicator B-15, Branson Sonfier, Kebo Lab AB). The significance of well-controlled pH and NaHCO₃ concentrations in the lysing buffer solution in obtaining reproducible results in irradiated samples as reported by Feinendegen *et al.* (1984) was confirmed in our studies on EAT cells growing *in vivo*. TK activity was then determined as described by Skog & Tribukait (1990). TK activity was expressed as mole dTdR phosphorylated to dTMP per min. Because the enzyme activity was mainly found in S-phase and G₂ cells (He *et al.* 1991), the enzyme activity was related to the number of S-phase and G₂ cells. The TK activity was also expressed as mole dTdR phosphorylated to dTMP per min and mg protein. The protein content of the cell extract was determined according to the method of Lowry *et al.* (1951). The standard deviation was no more than 10%.

Determination of activities in variant TKs

Variants of TK1 and TK2 were separated by an analytical thin-layer isoelectric focusing system (IEF) at a pH gradient of 3.5–9.0 as previously described (He *et al.* 1990). After focusing, the gel was cut into 2-mm pieces and TK activity was determined as described above. The pH was measured directly on the surface of the gel using a LKB-2117–111 Multiphor Electrode (Amersham Pharmacia, Uppsala, Sweden).

Three variants of TK1 with different pI values were found. The two major fractions found at pI 8.3 and 6.9 were denoted as TK1a and TK1b. TK1a represents a dephosphorylated form, while TK1b represents a phosphorylated form (He *et al.* 1996a). Using a broad pH gradient (3.5–9.0), a small peak was previously found at pI 5.3 containing a mixture of TK1 and TK2. These variants were denoted as TK-heterogeneous (TK-h) (He *et al.* 1990). Because the activity at pI 5.3 did not vary during the cell cycle, at least in tumour cells, while the activities at pI 6.9 and 8.3 were low in G₁ but high in S-phase and G₂ cells (He *et al.* 1991), the activity at pI 5.3 was expressed per cell while the activities at pI 6.9 and 8.3 were expressed per S-phase + G₂ cell. In the mouse adult liver cells, the peak at pI 5.3 was further separated into two peaks using a narrow pH gradient (pH 4–6), corresponding to pI 5.1 and 5.3, containing TK2 and TK1, respectively (He *et al.* 1990). The activities of TK2 and TK1 at pI 5.1 and 5.3 were expressed per mg protein.

Determination of the amount of TK1 enzyme by Western immunoblotting

The procedure of SDS electrophoresis and electrophoretic transfer was carried out as described by Pharmacia LKB Biotechnology (Manual, Pharmacia LKB 1989, Amersham Pharmacia), with slight modifications (He *et al.* 1996a). 10 µl of cell extract (1×10^5 cells) in SDS sample buffer [2% SDS, 10% glycerol (w/v), 2% β-mercaptoethanol (v/v) and 0.01% bromphenol blue] was analysed on a 4–20% gradient SDS-PAGE (Pharmacia LKB 2117 Multiphor II Electrophoresis System, Amersham Pharmacia). Standard proteins were run in parallel.

After electrophoretic transfer to a PVDF membrane, immunoblotting of the membrane was performed according to the ECL Western blotting protocol (Amersham Pharmacia), with slight modifications (He *et al.* 1996b). The membrane was stained with the anti-TK1 polyclonal antibody (pAb1, 1 mg IgG/ml) diluted 1 : 10 000, produced in our laboratory (He *et al.* 1996b). pAb1 was raised against the C-terminus of the human TK1 and recognizes both human and mouse TK1 (He *et al.* 1996b). This band was quantified by a Laser Densitometer (LKB, Ultrascan XL, Amersham Pharmacia). The values were expressed as AU × mm percentage/S + G₂ cell.

RESULTS

Cell cycle kinetics

The cell cycle compositions of the non-irradiated mouse spleen, thymus and liver cells are shown in Table 1. No significant changes in the cell cycle composition of spleen, thymus and liver cells were found up to 6 h after x-irradiation at doses of 0.01–5 Gy (results not shown). The cell cycle composition of the ascites tumour cells at day 4 after transplantation was: G₁ = 50%, S-phase = 40% and G₂ + M = 10%. Because of disturbance of cell cycle composition by irradiation, irradiation-induced changes in TK activity were corrected for cell cycle composition changes. The cell cycle composition was determined up to 24 h after irradiation. No change was found after 1 Gy. After doses of 3 and 5 Gy, the cells were continuously accumulated in G₂ with a corresponding decrease in the number of G₁ cells. The proportion of S-phase cells was unchanged except at 12 h, at which time it increased significantly (Table 1).

TK activity of spleen cells

The TK activity of the non-irradiated spleen was $17 \pm 0.5 \times 10^{-18}$ mol dTMP/S-phase + G₂ cell/min or $72 \pm 2 \times 10^{-12}$ mol dTMP/mg protein/min (Table 2). The relative activities of the TK1

Table 1. Cell cycle distribution of non-irradiated spleen, thymus, adult liver cells and mouse tumour cells (EAT), and of irradiated (5 Gy) EAT cells. Mean values of four animal ± SE are given

Cells	Time (h)	%G ₁	%S-phase	%G ₂ + M
Spleen	0	85 ± 2	12 ± 2	3 ± 1
Thymus	0	81 ± 3	13 ± 2	6 ± 1
Liver	0	72 ± 2	—	28 ± 1
EAT	0	50 ± 1	40 ± 2	10 ± 2
	1	45 ± 1	44 ± 3	11 ± 2
	3	41 ± 3	44 ± 5	15 ± 2
	6	35 ± 3	50 ± 2	15 ± 2
	12	15 ± 4	58 ± 4	27 ± 2
	24	27 ± 5	40 ± 3	33 ± 3

Table 2. Activities of TK1 and TK2 in mouse EAT, spleen, thymus and adult liver cells. Three variants of TK1: TK1a, TK1b and TK-h in EAT, spleen and thymus at pI values of 8.3, 6.9 and 5.3, respectively, were found. Two isoenzymes, TK2 and TK1, at pI values of 5.1 and 5.3 were found in liver cells. The activity was expressed as thymidine phosphorylated to dTMP per min. Mean values $\pm$ SE of three or four animals are given

Cells	Total activity		Activity of TK1 variants			TK2
	/S + G ₂ cells ($\times 10^{-18}$)	/mg protein ($\times 10^{-12}$)	At pI 8.3	6.9	5.3	5.1
			/S + G ₂ cells ($\times 10^{-18}$)	/S + G ₂ cells ($\times 10^{-18}$)	/cells ($\times 10^{-18}$)	/mg protein ($\times 10^{-12}$)
EAT	33 $\pm$ 2	175 $\pm$ 10	17.8 $\pm$ 0.5	8.8 $\pm$ 0.3	2.5 $\pm$ 0.3	
Spleen	17 $\pm$ 2	72 $\pm$ 2	2.8 $\pm$ 0.3	8.4 $\pm$ 2.4	2.1 $\pm$ 0.2	
Thymus	18 $\pm$ 2	83 $\pm$ 11	4.9 $\pm$ 1.4	7.0 $\pm$ 0.7	2.8 $\pm$ 0.2	
Liver	—	5 $\pm$ 0.5	—	—	0.04 $\pm$ 0.01*	1.0 $\pm$ 0.04

*Expressed per mg protein $\times 10^{-12}$.

Table 3. Activities of TK1a, TK1b, TKh and TK2 of spleen, thymus and adult liver from mouse after irradiation. TK activity of spleen cells was measured 1 (0.5 Gy) and 3 (1 Gy) h after irradiation, while TK activity of thymus was measured 3 h (1 Gy) and TK activity in liver cells was measured 3 h (5 Gy) after irradiation. Activities were expressed as dpm/cell/min and in percentage of non-irradiated cells. Mean values $\pm$ SE from five experiments are given

Dose (Gy)		TK1a	TK1b	TK-h1	TK2
0.5	Spleen	57 $\pm$ 10	29 $\pm$ 1	102 $\pm$ 13	
1		46 $\pm$ 11	92 $\pm$ 18	40 $\pm$ 5	
1	Thymus	44 $\pm$ 2	40 $\pm$ 7	15 $\pm$ 4	
5	Liver	—	—	133 $\pm$ 17	113 $\pm$ 11

variants TK1a, TK1b and TK-h of non-irradiated spleen cells are shown in Table 2. TK1a represents 21%, TK1b 63% and TK-h 16%. The ratio between the phosphorylated TK1b and the dephosphorylated TK1a was 3.0.

The total TK activity after irradiation with doses between 0.01 and 1 Gy up to 6 h is shown in Fig. 1. The graph of TK activity with time after irradiation (Fig. 1a) reveals a rather complicated picture with an increase in TK activity at the lowest dose and a decrease at higher doses. In a dose-response graph of the values at the various times of measurement (Fig. 1b), the independence of the changes with time is clearer. Thus, in the low dose range of 0.01–0.05 Gy, most values were either unchanged or elevated. A breakpoint at 0.1 Gy was found at which the activity was about 50% of the normal value. At higher doses, the activity was low and almost dose-independent.

The activities of TK1s in the irradiated spleen were measured 1 h after a dose of 0.5 Gy and 3 h after a dose of 1 Gy (Table 3), at which times the total TK activity was reduced by about 60% (Fig. 1). Irradiation with a dose of 0.5 Gy decreased the activities of TK1a and TK1b by 40 and 70%, respectively, but did not affect TK-h. Irradiation with a dose of 1 Gy resulted in a decrease in the activities of TK1a and TK-h, but not TK1b. The TK1b/TK1a ratio decreased to 0.5 after 0.5 Gy, but increased to 2.0 after 1 Gy, demonstrating considerable changes in the degree of phosphorylation in addition to the changes in total enzyme activity.

TK activity of thymus cells

The enzyme activity of non-irradiated thymus cells was $18 \pm 2 \times 10^{-18}$ mol dTMP/S-phase + G₂ cell/min or $83 \pm 11 \times 10^{-12}$ mol dTMP/mg protein/min and very similar to that of spleen cells

Figure 1. (a) TK activity of spleen cells up to 6 h after irradiation doses of 0.01 (□), 0.05 (●), 0.1 (▲), 0.5 (△) and 1 (○) Gy. (b) TK activity of spleen cells at 1 (●), 3 (○) and 6 (★) h vs. dose of irradiation. The values are expressed as $\times 10^{-18}$ mol dTdr phosphorylated to dTMP/S + G₂ cell/min and as percentages of the values for non-irradiated cells. Mean values $\pm$ SE for four animals are shown.

(Table 2). The relative activities of the TK1a, TK1b and TK-h of non-irradiated thymus cells were 33, 48 and 19%, respectively, and about the same as found in spleen cells (Table 2). The TK1b/TK1a ratio of 1.4 indicates that the phosphorylated form is lower in thymus than in spleen cells.

The thymus was irradiated with doses of between 0.01 and 1 Gy. The results of the changes in TK activity with time demonstrate an increase after a dose of 0.1 Gy and a decrease after both lower and higher doses than 0.1 Gy (Fig. 2a). The time-independence is further illustrated in a dose-response curve for the values at various times (Fig. 2b). This graph demonstrates profound differences in the reaction between spleen and thymus. Three hours after an irradiation dose of 1 Gy, the activities of TK1a, TK1b and TK-h decreased by 60–85% (Table 3). No change in the TK1b/TK1a ratio was found.

Figure 2. (a) TK activity of thymus cells up to 6 h after irradiation doses of 0.01 (□), 0.05 (●), 0.1 (▲), 0.5 (△) and 1 (○) Gy. (b) TK activity of thymus cells at 1 (●), 3 (○) and 6 (★) h vs. irradiation doses (Gy). The values are expressed as $\times 10^{-18}$ mol/S + G_2 /min and as a percentage of the values for non-irradiated cells. Mean values $\pm$ SE for four animals are shown.

The dramatic fluctuation of TK activities after X-irradiation was also found by Feinendegen *et al.* (1984). They found an extremely high sensitivity and fluctuation of TK activity in mouse bone marrow cells after low-dose exposure (0.01–0.1 Gy) to ionizing radiation (see Fig. 3).

TK activity of liver cells

The TK activity of the liver cells was only expressed per mg protein, since the adult liver did not contain proliferating S-phase cells while 28% of the cells were tetraploid. The TK activity was $5 \pm 0.5 \times 10^{-12}$ mole dTMP/mg protein/min. This corresponds to 6–7% of the activity present in thymus and spleen (Table 2) and 3% of the activity present in EAT cells. In Table 2, the majority of the activity was found as TK2 (96%) at pI value of 5.1, while only 4% corresponded to TK1 in cytoplasm (pI 5.3), as previously described by He *et al.* (1990, 1996a).

After irradiation with a dose of 5 Gy, the total TK activity increased at 3 h by about 60% and then decreased back to normal values at 6 h (data not shown). The two isoenzymes reacted in similar ways (Table 3).

Figure 3. TK activities of mouse bone marrow cells as reported by Feinendegen *et al.* (1984). (a) Relation to time at dose 0.1 (○) and dose 1.0 (●) and (b) to irradiation at dose 0.1 (○), 4 h after X-irradiation. Mean values $\pm$ SD are shown.

TK activity and the cellular amount of TK1 in mouse tumour cells

The TK activity of non-irradiated tumour cells growing *in vivo* was $33 \pm 2 \times 10^{-18}$ mol dTdr phosphorylated to dTMP/S + G₂ cell/min or $175 \pm 10 \times 10^{-12}$ mol dTdr phosphorylated to dTMP/mg protein/min (Table 2). The relative activities of variants of TK1a, TK1b and TK-h were 61, 30 and 9%, respectively (Table 2).

No significant changes in the TK activity were found after *in situ* X-irradiation with 0.1 Gy. Irradiation at 1, 3 and 5 Gy had similar effects on TK activity (Fig. 4), which decreased to a minimum value after 1 h, and increased to a maximum at 3 h, but decreased again to a new minimum value at 6 h. At 12 h, 80–90% of the initial values were reached. At 24 h, the TK activity of cells irradiated with 3 and 5 Gy decreased again to about half of the initial activity. Thus, with a few exceptions (5 Gy, 30 mins and 1 Gy, 24 h), the inhibition of TK activity was independent of the dose given above 0.1 Gy.

TK1 activity of the three variants after *in situ* irradiation with 5 Gy was studied up to 24 h (Fig. 5). TK1b followed the dramatic oscillation of TK1a with minimum values at 1, 6 and 24 h, and the ratio between the phosphorylated and the dephosphorylated TK1 activities was unchanged (Table 4). TK-h decreased significantly by 50% at 6 h but normalized between 12 and 24 h.

The growth of tumour cells in the culture medium was also studied. The TK activity of control cells was $10\text{--}20 \times 10^{-18}$ mol dTdr phosphorylated to dTMP/S + G₂ cell/min, depending

Figure 4. Total TK activity of tumour cells after *in situ* irradiation with doses of 0.1 ($\square$), 1 ($\blacktriangle$), 3 ($\triangle$) and 5 ($\circ$) Gy. The activities ($\times 10^{-18}$ mol dTMP/S + G_2 cell/min) are expressed as a percentage of the values of non-irradiated cells. Mean values $\pm$ SE of four to eight animals are shown.

Figure 5. The activities of variants of TK1 in tumour cells after *in situ* irradiation with a dose of 5 Gy. The values of TK1a ($\circ$) and TK1b ($\blacksquare$) are expressed as $\times 10^{-18}$ mol dTMP/S + G_2 cell/min, while the value of TK-h ($\triangle$) is expressed as $\times 10^{-18}$ mol dTMP/cell/min. Mean values $\pm$ SE of four to eight animals are shown.

on the growth conditions of the cells. Following a dose of 5 Gy, minimum activity values at 1 and 6 h and maximum activity at 3 h were found, similar to the results after *in situ* irradiation (data not shown). However, 2.5 Gy resulted, after an early transient increase, only in a decrease of TK activity (data not shown). The increase in TK activity by 30 min after 2.5 Gy was due to an increase of both TK1a and TK1b (data not shown).

Table 4. Ratio between the phosphorylated (TK1b) and the dephosphorylated (TK1a) forms of TK1 of EAT cells following an *in situ* irradiation dose of 5 Gy. Mean values of 4–8 animals are given

Time (h)	TK1b/TK1a
Control	0.5 ± 0.020
0.5	0.5 ± 0.038
1	0.6 ± 0.078
3	0.4 ± 0.034
6	0.4 ± 0.050
12	0.5 ± 0.036
24	0.4 ± 0.065

Figure 6. Amount of TK1 polypeptide as determined by Western immunoblotting as a percentage of non-irradiated cells after *in situ* irradiation with a dose of 5 Gy. 10 µl of cell extract containing 10^5 cells was injected into the well. Western immunoblotting is shown in the inset. The final values are expressed as a percentage of non-irradiated cells per S-phase + G₂. Mean values ± SE of six animals are shown.

The cellular amount of TK1 was determined using anti-TK1 polyclonal antibody (pAb1). The amount of TK1 enzyme after irradiation with 5 Gy was unchanged up to 12 h, but decreased by about 50% at 24 h (Fig. 6).

DISCUSSION

In this study, we investigated the total TK activity, and the activity of the isoenzymes TK1 and TK2, and isoenzyme TK1 variants in four different cell systems representing growing (mouse spleen, thymus and tumour) and resting (liver) cells.

The proliferation activities of these cell systems were related to the proportions of cells in S-phase and G₂, amounting to about 15% in spleen and thymus, and about 50% in the tumour cells. No S-phase was present in the adult liver, while 28% of the cells were tetraploid. The TK activity was consistent with the differences in proliferation rate with high activities of TK1 in

spleen, thymus and tumour and almost no activity of TK1 in the liver. The cell material from thymus, liver and tumour was most homogeneous. Like cell material from bone marrow, cells from the spleen contain a mixture of different haematopoietic cells, which may complicate the interpretation of the reaction of TK to irradiation. The presence of various TKs further complicates the interpretation of the results. Two different isoenzymes (TK1, TK2) were found, of which TK1 appeared in two different forms according to its pI values: TK1a = 8.3 and TK1b = 6.9, representing dephosphorylated and phosphorylated forms of TK1 (He *et al.* 1991, 1996a). TK1 and TK2 have previously been characterized by kinetic studies with different concentrations of thymidine (dTdR), adenosine triphosphate (ATP) and deoxythymidine triphosphate (dTTP) and also by their abilities to use various substrates and phosphor donors (He *et al.* 1990). To exclude the possibility that irradiation-induced changes in the cell cycle composition were the reason for the changes in the TK activity, all results were corrected for such deviations.

The very high sensitivity to irradiation found by Feinendegen and co-workers (Feinendegen *et al.* 1984; Hohn-Elkarim *et al.* 1990) in bone marrow cells was mainly confirmed by our study in spleen and thymus cells. The activity of TK was rapidly reduced to about 50% and then continued to be inhibited at this level even if the dose was elevated, as reported by Feinendegen and co-workers. However, the bone marrow cells were 10 times more sensitive than the spleen cells and 100 times more sensitive than the thymus cells, at 4–6 h after irradiation, in terms of the TK activities.

Considering various irradiation doses and times after irradiation at which the TK activity was measured, and comparing the reactions of the various tissues, it is obvious that the effect of radiation on TK is more complicated than would be expected from earlier results in bone marrow cells. In spleen, minimum values of TK1 were observed after 0.1 Gy. Increases in dose did not result in further inhibition of activity. The significant increase 1 h after a dose of 0.01 Gy indicates the existence of both activating and inactivating processes in a complex interplay. This complex interplay is further demonstrated by the results from thymus in which TK activity, after an initial decrease at 0.01–0.05 Gy, reached a supernormal maximum at 0.1 Gy. The dramatic decline of the activity of the dephosphorylated TK1 (TK1a), and the differences in the changes in the degree of phosphorylation of TK1 after irradiation between spleen and thymus, further underline the complexity of the response of the enzyme activity to irradiation. In the liver, which contains mainly TK2, only increased values of TK activity were observed.

In contrast to these results in spleen and thymus, the present results in ascites tumour cells demonstrate a quite different behaviour: most remarkable were the oscillating changes of TK activity, with maximum values 3 and 12 h following irradiation after minimum values at 1 and 6 h. These values were dose-independent in the dose range of 1–5 Gy. Irradiation with a dose of 0.1 Gy did not result in significant changes of TK activity, indicating lower radiosensitivities of the ascites tumour cells than found in spleen and thymus, as well as in bone marrow cells. As 96% of the TK activity in the liver belongs to the mitochondrial TK2 and only $\approx 3\%$ of the total TK activity in tumour cells belongs to TK2 (Table 3), the possible influence of TK2 in our measurements of the total TK activity is obviously negligible.

Considering the degree of proliferation as an important factor in the irradiation response of TK, ascites tumour cells in exponential growth, as used in our experiments, had the highest fraction of proliferating S-phase cells: about 40% compared with 12 and 13% in spleen and thymus. Corresponding values for total TK activity (expressed as thymidine phosphorylated to dTMP/min) were 33×10^{-18} , 17×10^{-18} and 18×10^{-18} per S-phase and G₂ cell, respectively, or double in the ascites tumour cells compared to spleen and thymus. Hence, in the ascites tumour, both the fraction of cells in the S-phase and G₂, and the activity of TK, expressed per S-phase and G₂

cell, were doubled. Furthermore, the irradiation-induced decrease in TK activity by about 50% was the same in all cell systems studied, including the bone marrow as reported by Feinendegen *et al.* (1984). Thus, the irradiation response of TK activity is obviously not directly linked to the extent of proliferation, but follows the well-known differences in radiosensitivity of lymphatic tissue, on the one hand, and epithelial tumour cells, on the other hand.

By isoelectric focusing, we identified two major variants of the cytoplasmic TK1 and, separately from these, the mitochondrial TK2. In agreement with findings in spleen, the two variants of TK1 reacted in similar ways upon irradiation, with an unchanged ratio of TK1a and TK1b, irrespective of high or low total TK activity. Since these variants represent phosphorylated and dephosphorylated forms of TK1 (He *et al.* 1996a), any irradiation effect on the degree of phosphorylation of TK1 can be excluded. The dramatic change in dephosphorylated TK1 (TK1a and b) activity in mouse spleen, thymus and mouse ascites tumour may suggest that TK1a and TK1b are more radiosensitive than TK-h.

Cell cycle related studies of the TK1 polypeptide and TK1 activity showed a close relationship to the cell cycle, with low values in G₁, high values in S-phase, and decreasing values in G₂ of the cell cycle (He *et al.* 1991). Therefore, the amount of TK1 polypeptide was expressed per S and G₂ cell. In spite of profound changes in TK1 activity following irradiation up to 12 h, the amount of TK1 polypeptide was unchanged after irradiation; it decreased in the first 24 h post-irradiation by about 50%. The amount of TK1 polypeptide was determined by an affinity purified polyclonal anti-TK1 antibody raised against a synthetic peptide corresponding to part of the C-terminus of human TK1. This antibody recognizes a 25-kDa cytoplasmatic polypeptide of TK1, which is lacking in resting cells or TK-negative mutants. Since only the dephosphorylated TK1 is recognized by this antibody (He *et al.* 1996a), the unchanged degree of phosphorylation of the TK1 was an important prerequisite for a reliable quantification of the TK1 polypeptide.

Studies on the activity and protein synthesis of the TK enzyme further support the assumption that a direct irradiation effect on the enzyme is not responsible for the irradiation-induced changes in TK activity. In a previous study on mouse ascites tumour cells we showed that TK activity and the amount of TK1 polypeptide were closely related to the cell cycle, i.e. low in G₁, increasing during the S-phase and decreasing in G₂ (He *et al.* 1991, 1996b). Thus, TK activity in these cells is related to the amount of TK when growing normally. We also found that the half-life of TK was short (45 min) (He *et al.* 1991). An inhibition of TK1 polypeptide synthesis by irradiation should therefore easily be recognized, but was not observed, at least up to 12 h. On the contrary, we showed in paper II of this series (He *et al.* 2001) that the reduced TK1 activity is mainly due to an elevated intracellular concentration of dTTP. The decreases in the amount of TK1 polypeptide and its activity 24 h after irradiation found in this study, at least, were due to the accumulation of cells in G₂ (irradiation induced G₂-arrest), a cell cycle stage where the amount of TK1 polypeptide and thus its activity decrease. The decreases in the amount of TK1 in G₂ were due to the rapid degradation of TK1 polypeptide rather than deactivation of the enzyme. The reduced TK activity at 24 h after irradiation is also caused by the elevated number of dead cells appearing at this time point (Skog & Tribukait 1984).

In conclusion, activation and inactivation of TK1 at low doses of irradiation in spleen and thymus make it difficult to determine general mechanisms behind the interaction between irradiation and TK1 activity. The individual reactions of TK1 and TK2 in various organs suggest the significance of specific environmental factors in the expression of the irradiation effect. It seems that lower doses interact mainly with regulatory events of TK activity, while higher doses affect TK indirectly by general cell damage. The significance of changes in TK1 activity for deoxynucleotide metabolism is the subject of further studies.

ACKNOWLEDGEMENTS

We sincerely thank Taylor and Francis Ltd for permission to reproduce Fig. 3 from Feinendegen *et al.* (1984) (<http://www.tandf.co.uk/journal>). This study was supported by the Stockholm Cancer Society and King Gustav V's Jubilee foundation.

REFERENCES

- ARNÉR E, ERIKSSON S (1995) Mammalian deoxyribonucleoside kinases. *Pharmacol. Ther.* **67**, 155.
- FEINENDEGEN LE, MÜHLENSIEPEN H, LINDBERG C, MARX J, PORSCHEN W, BOOZ J (1984) Acute and temporary inhibition of thymidine kinase in mouse bone marrow cells after low-dose exposure. *Int. J. Radiat. Biol.* **45**, 205.
- HE Q, SKOG S, TRIBUKAIT B (1991) Cell cycle related studies on thymidine kinase and its isoenzymes in Ehrlich ascites tumours. *Cell Prolif.* **24**, 3.
- HE Q, SKOG S, WELANDER I, TRIBUKAIT B (1990) Enzyme kinetics of thymidine kinase isoenzymes of Ehrlich ascites tumour. *Anticancer Res.* **10**, 1257.
- HE Q, SKOG S, WELANDER I, TRIBUKAIT B (2001) X-irradiation effects on thymidine kinase (TK): II. The significance of deoxythymidine triphosphate for inhibition of TK1 activity. *Cell Prolif.* **35**, 83.
- HE Q, SKOG S, WU J, JOHANSSON A, TRIBUKAIT B (1996a) A phosphorylated and dephosphorylated form of cytosolic thymidine kinase (TK1) in mouse Ehrlich ascites tumour cells. *Biochim. Biophys. Acta* **1289**, 25.
- HE Q, WANG N, SKOG S, ERIKSSON S, TRIBUKAIT B (1996b) Characterization of a peptide antibody against a C-terminal part of human and mouse cytosolic thymidine kinase, which is a marker for cell proliferation. *Eur. J. Cell Biol.* **70**, 117.
- HOHN-ELKARIM K, MÜHLENSIEPEN H, ALTMAN KI, FEINENDEGEN LE (1990) Modification of effects of radiation on thymidine kinase. *Int. J. Radiat. Biol.* **58**, 97.
- LOWRY OH, ROSEBOURGH NY, FASS AC, RANDELL RJ (1951) Protein measurements with the folin phenol reagent. *J. Biol. Chem.* **193**, 265.
- SKOG S, TRIBUKAIT B (1984) Irradiation induced cell death as related to the cell cycle, *Acta Radiol. Oncology* **24**, 1.
- SKOG S, TRIBUKAIT B (1985) Analysis of cell flow and cell loss following X-irradiation using sequential investigation of the total number of cells in the various parts of the cycle. *Cell Tissue Kinet.* **18**, 427.
- SKOG S, HE Q, TRIBUKAIT B (1990) Lack of correlation between thymidine kinase activity and changes of DNA synthesis with tumour age. An *in vivo* study in Ehrlich ascites tumour. *Cell Tissue Kinet.* **23**, 603.
- TRIBUKAIT B, MOBERGER G, ZETTERBERG A (1975) Methodological aspects of rapid-flow cytofluorometry for DNA analysis of human urinary bladder cells. In: Haanen CAM, Hillen HFP, Wessels JM, eds. *Pulse-Cytophotometry I. First International Symposium on Pulse Cytophotometry*, p. 50. Ghent: European Press Medikon.