X-irradiation effects on thymidine kinase (TK): II. The significance of deoxythymidine triphosphate for inhibition of TK1 activity

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Abstract. The purpose of this study was to investigate the mechanism behind the high sensitivity of thymidine kinase 1 (TK1) to X-irradiation. The deoxythymidine triphosphate (dTTP) pool was studied in mouse ascites tumour cells 1–24 h after X-irradiation with 5 Gy. Irradiation changed the Michaelis-Menten kinetics of TK1 from linear to biphasic, showing a negative co-operativity. These changes were closely related to changes in the dTTP pool. Addition of dTTP to the cell extract of non-irradiated cells, or thymidine (dTdR) to the culture medium, resulted in changes very similar to the kinetics found in the irradiated cells. Addition of 5 ϕ -amino-5 ϕ -deoxythymidine (5 ϕ -AdTdR), a thymidine analogue that eliminated the inhibitory effect of dTTP on TK1 activity, completely abolished the irradiation-induced inhibition of TK1 activity. We suggest that the reduced TK1 activity is mainly due to an elevated intracellular concentration of dTTP.

INTRODUCTION

In paper I in this series, we carried out studies on the X-irradiation effect on thymidine kinase 1 (TK1) in mouse spleen, thymus and ascites tumour cells. The results showed complicated patterns. 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. In ascites tumour cells, following doses of between 1 and 5 Gy, a dose-independent decrease by about 30% 1 h after irradiation was followed by subnormal maximum values at 3 h, minimum and maximum values at 6 and 12 h, and further minimum values at 24 h. TK1 variants, representing different degrees of phosphorylation, reacted in a similar way to the total TK activity, and, thus, no change in the degree of phosphorylation of TK1 was found. The amount of TK1 polypeptide in Western immunoblotting did not reveal any change of the TK1 polypeptide up to 12 h after irradiation. The decrease in TK1 activity found at 24 h could, however, be explained by a corresponding decrease in the amount of TK1 polypeptide.

Earlier pioneering work by Feinendegen and co-workers (Feinendegen *et al.* 1984, 1988; Hohn-Elkarim *et al.* 1990) on thymidine kinase in bone marrow cells also showed independence

within the dose-range of 0.01–1 Gy, but a relatively long time to reach maximum inhibition, which disappeared after 10 h.

In previous studies using ascites tumour cells we found that the intracellular amount of deoxythymidine triphosphate (dTTP) was elevated 1–2 h after X-irradiation with a dose of 5 Gy (Skog & Tribukait 1989). Since TK1 activity is feedback-inhibited by dTTP (Bresnick *et al.* 1964; Ellims *et al.* 1981; Barrie *et al.* 1984), the irradiation-induced decrease of TK1 activity could be due to the inhibitory effect of dTTP.

Therefore, in this study in ascites tumour cells we measured the size of the cellular dTTP pool following irradiation and related it to the changes in TK1 activity. These changes in TK1 activity corresponded well to those found when the dTTP pool of non-irradiated cells was manipulated by changing the extracellular dTdR concentration. In an *in vitro* assay, the Michaelis-Menten kinetics of TK1 activity after *in situ* irradiation was studied at various concentrations of dTdR and dTTP. The shift from a linear kinetics in non-irradiated cells to a biphasic kinetics after irradiation was consistent with the biphasic kinetics found when the TK1 activity of non-irradiated cells was inhibited by dTTP. In this context, the effect of NaHCO₃ on the Michaelis-Menten kinetics of TK1 activity was also studied. To further investigate the involvement of dTTP in the irradiation-induced inhibition of TK1 activity we used 5'-AdTdR, a dTdR analogue, which is able to abolish the inhibitory effect of dTTP on TK1 activity.

The sum of all observations supports the assumption that dTTP is the main factor for the irradiation-induced changes of TK1 activity.

MATERIALS AND METHODS

Cells

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

Ehrlich ascites tumour cells growing *in vivo* were removed from the animals 4 days after transplantation, washed in Tris buffer (Tris 0.1 m, NaCl 0.07 m, and EDTA 0.005 m, pH 7.5) and 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.

Irradiation

X-irradiation was performed at 250 kV, 15 mA, 0.5 mm Cu filtration, SSD 50 cm and 1.33 Gy/min. The dosimetry was based on lithium fluoride dosimetry and checked routinely using a Simplex Universal dosimeter (Kebo Lab AB, Stockholm, Sweden).

Tumour cells growing *in vivo* were irradiated *in situ* 4 days after transplantation at exponential growth, while cells growing *in vitro* were irradiated in the culture medium at 37 °C, 20–24 h after suspending the cells in the medium when they were growing exponentially.

Cell cycle composition

The cell cycle composition was determined by flow-cytometry as described previously (Tribukait *et al.* 1975). The proportions of cells in the various cell cycle phases (G₁, S-phase and G₂ + M)

were determined from the different areas of the histograms assuming a Gaussian function of the G_1 and $G_2 + M$ peaks and attributing the remaining part of the DNA histogram to cells in the S-phase. The proportions of S-phase cells measured were in good agreement with the ^3H -dTdR labelling index (Skog & Tribukait 1985).

Determination of TK1 activity in cytoplasmic fraction

Cells growing *in vivo* were removed from the animal and suspended in cold Tris buffer (100 mM Tris, 70 mM NaCl, and 5 mM EDTA, pH 7.5) and washed twice. Cells were then resuspended to a final concentration of 5×10^6 cells/ml in a lysing buffer containing 10 mM Tris buffer, pH 7.5; 0.5% P-40 (V/V); 2 mM β -mercaptoethanol; 5 mM NaF; 5 mM MgCl_2 , with or without various concentrations of NaHCO_3 and with different pH values, depending on experimental conditions (see Results). *In vitro* cells were resuspended to a final concentration of 5×10^6 cells/ml in the above-mentioned lysing buffer. The cytoplasmic fraction was obtained by centrifugation at 4000 *g* and then the supernatant was collected after centrifugation at 48 000 *g*. TK1 activity was determined according to the previous method (Skog *et al.* 1990). Fifty microlitres of the supernatant of the cell extract was added to 200 μl of cocktail (55 mM Tris-HCl, pH 7.5, 5.6 mM NaF, 5.0 mM ATP, 3.8 mM MgCl_2 , and 0.06 mM unlabelled dTdR, unless otherwise stated in the Results) and 85.1 kBq or 2.3 μCi [^3H]-dTdR (specific activity 0.74 TBq or 20 Ci/mmol, New England Nuclear, Boston, MA) and incubated for 15 min at 37 °C. The activity was expressed as disintegration per minute (dpm)/S + G_2 cells/min. TK1 activity was expressed as S + G_2 cell since the contribution of activity from G_1 cells is low (He *et al.* 1990). The standard deviation was no more than 10%.

In paper I of this series, at these time points, minimum TK1 activity values were found at 1, 6 and 24 h, while subnormal maximum values were observed at 3 and 12 h. The fluctuation of TK1 activity during the time of radiation is very difficult to understand. In the present paper, dTdR kinetics of TK1 was studied according to the method of Munch-Petersen *et al.* (1984, 1993). For the determination of the effect of dTTP on dTdR substrate kinetics, 50 μl of cell extract in non-irradiated cells was added to 200 μl of cocktail with varying concentrations of dTdR between 1 and 80 μM at a dTTP concentration of 20, 30 and 40 μM and a constant ATP concentration of 5 mM. For the determination of the radiation effect on dTdR substrate kinetics, 50 μl of cell extract of irradiated cells was added to 200 μl of cocktail containing varying dTdR from 1 to 80 μM at a constant ATP concentration of 5 mM. The Hofstee plot for the determination of the Michaelis-Menten constant (K_m), the maximum velocity (V_{max}) and the Hill constant were calculated as described by Munch-Petersen *et al.* (1993).

In the experiments in which the effect of dTTP on TK1 activity was modified by a dTdR analogue, 5'-AdTdR (0–300 μM , Sigma, St. Louis, MO) (Fischer & Baxter 1985) was added either to the cell extract of *in vivo* growing cells or to the culture medium when the cells were growing *in vitro*.

Measurements of dTTP

The cells were incubated for 1.5 h in culture medium and after further incubation for 30 min in the presence of 37 kBq or 1 μCi [^3H]-dTdR (specific activity 0.74 TBq/mmol, New England Nuclear), excess cold Tris buffer was added. The cells were centrifuged at 4800 *g* at 4 °C, resuspended in an appropriate volume and then centrifuged at 14 400 *g* at 4 °C. The cells were precipitated in 0.3 M TCA and after 20 min neutralized with 0.5 M freon-acetylamine (1 : 1.1) to a pH of about 5.0. The amount of dTTP was then analysed by high-performance liquid chromatography (HPLC) on a Whatman Partisil 10 anion column (Waters, Milford, MA) using 0.4 M ammoniumphosphate buffer containing 5% acetonitril, pH = 3.4. The flow rate of the buffer stream was 2.0 ml/min. The peak corresponding to dTTP was identified by the retention time, the ratio between the area of the dTTP peak measured at 254 and 280 nm, respectively, and by

Figure 1. (a) Hofstee plots of the effect of radiation on dTdR kinetics. A plot of V vs. $V/[S]$ has a slope of K_m . Non-irradiated cells (■) and of cells at 1 (△), 3 (●), 6 (▲) 12 (○) and 24 (□) h after *in situ* irradiation with a dose of 5.0 Gy are represented. (b) Hill plots are shown in the inset. V (TK1 activity) is expressed as dpm/ 10^3 S + G₂ cells/min. Mean values of four mice are shown.

internal standard (Skog *et al.* 1990). The peak height was estimated by means of a computer program (Drew Scientific, London).

RESULTS

The effect of radiation on thymidine substrate kinetics of TK1

The dTdR kinetics of TK1 in the irradiated cells was studied 1, 3, 6, 12 and 24 h after *in situ* irradiation in the dTdR concentration range of 1–60 μM (Fig. 1). The non-irradiated control cells showed a linear Michaelis-Menten kinetics by Hofstee plot, in agreement with an earlier study (He *et al.* 1990). In contrast, the dTdR kinetics of TK1 in the irradiated cells, irrespective of time after irradiation, changed to biphasic kinetics (Fig. 1), which indicated a negative co-operative kinetics as described previously (Munch-Petersen *et al.* 1993). The Hill constant decreased by about 50% up to 3 h and then increased to about 80–90% of the normal value (Table 1, Fig. 1).

Table 1. The Michaelis-Menten constant (K_m), maximum velocity (V_{max}) of TK1 and Hill constants (n) of TK1 in non-irradiated cells and irradiated cells after 1, 3, 6, 12 and 24 h at a dose of 5 Gy. The V_{max} (TK1 activity) is expressed as dpm/ 10^3 S + G₂ cells/min. Mean values for four to eight mice are given

Time (h)	K_m		V_{max}		Hill constant (n)
	K_m^1	K_m^2	V_{max}^1	V_{max}^2	
0		5.15		570	0 : 00
1	2.2	15.6	115	312	0.74
3	1.9	24.6	117	500	0.54
6	2.8	9.6	150	340	0.87
12	3.7	12.7	200	460	0.89
24	3.0	14.4	180	360	0.84

Figure 2. Hill constant (■) and dTTP pool (□) of cells irradiated with a dose of 5 Gy *in situ*. The values are expressed as a percentage of non-irradiated cells. Mean values of four animals are shown.

Figure 3. (a) Hofstee plot of the effect of dTTP on dTdR kinetics in non-irradiated cells in the presence of 0 μM (■), 20 μM (●), 30 μM (○) and 40 μM (△) dTTP at a fixed concentration of ATP of 5 mM. (b) Hill plots are shown in the inset. V (TK1 activity) is expressed as dpm/ 10^3 S + G_2 cells/min. Mean values of four mice are shown.

dTTP pool

The intracellular amount of dTTP of non-irradiated cells was 5×10^{-18} mol/cell. The dTTP pool was more than doubled 1 h after irradiation with 5 Gy and then decreased exponentially to normal levels at 24 h (Fig. 2).

Earlier results cell extract results suggest that the biphasic pattern of the TK1 kinetics found here in the irradiated cells is consistent with an inhibition of TK1 activity, which started to decrease at 10 μM dTTP (He *et al.* 1990). The physiological concentration of the intact cells was 10 μM dTTP, as calculated from the cellular amount of dTTP (5×10^{-18} mol/cell), a cellular volume of 6.5×10^{-13} l (Skog & Tribukait 1985) and a cellular water concentration of 75% of equal intracellular distribution. Thus, in this paper, we repeated these experiments at varying concentrations of dTTP (20–40 μM) in the cell extract. dTTP was again found to induce biphasic dTdR kinetics and the Hill constants were 0.48 at 20 μM and 0.18 at 40 μM (Fig. 3, Table 2).

In order to further demonstrate a relationship between TK1 activity and the intracellular concentration of dTTP, non-irradiated cells were incubated at varying concentration of dTdR

Table 2. The Michaelis-Menten constant (K_m), the maximum velocity (V_{max}) and Hill constants (n) of TK1 enzyme activity in cells growing *in vivo* exponentially in the presence or absence of dTTP. The V (TK1 activity) was expressed as dpm/10³ S + G₂ cells/min. Mean values for four mice are given

Conc. dTTP (μ M)	K_m		V_{max}		Hill constant (n)
	K_m^1	K_m^2	V_{max}^1	V_{max}^2	
0		5.4		459.0	1.0
20	2.0	9.0	120.0	190	0.48
30	3.3	37.5	60.0	190	0.59
40	2.5	42.5	13.5	150	0.18

Figure 4. Relationships between TK1 activity (■) and dTTP pool (□) for non-irradiated cells growing at different concentrations of dTdR in the medium. The values are expressed as the percentage of cells growing without dTdR. Mean values $\pm$ SE of four animals are shown.

for 2 h. As shown in Fig. 4, the increase in the concentration of the dTTP pool, which started at a concentration of 3 μ M dTdR in the medium, was mirrored by a corresponding decrease in TK1 activity.

Modification of TK1 activity by 5'-AdTdR

In this paper, the inhibitory effect of dTTP was further studied by addition of 5'-AdTdR, a thymidine analogue competing with dTTP. The concentration-dependent effect of 5'-AdTdR on TK1 activity was also studied in cell extracts from non-irradiated cells in the presence or absence of dTTP (Fig. 5). In the absence of dTTP, TK1 activity decreased gradually with increasing 5'-AdTdR concentrations. Addition of 20 μ M dTTP resulted in a decrease of TK1 activity by about 80%. This inhibition caused by dTTP was abolished when 5'-AdTdR was added up to 10 μ M (Fig. 5). Above this concentration, TK1 activity was reduced to the same extent as was found in the presence of 5'-AdTdR only.

In subsequent experiments, non-irradiated cells were grown *in vitro* at dTdR concentrations of 30 and 60 μ M, which almost doubled the intracellular concentration of dTTP, and in the presence or absence of 10 μ M 5'-AdTdR. In the absence of 5'-AdTdR, TK1 activity decreased by about 50% as compared with cells growing without dTdR. The addition of 5'-AdTdR to the culture medium completely restored TK1 activity (Table 3).

In the last experiment, the tumour cells were irradiated (5 Gy) *in situ*, transferred to culture medium after 1 h, and incubated for 30 or 60 min in the presence or absence of 10 μ M 5'-

Figure 5. The effect of 5'-AdTdr on TK1 activity in the presence (●) or absence (○) of 20 μM dTTP in the cell extract. TK1 activity is expressed as dpm/ 10^6 cells/min. Mean values $\pm$ SE of 4–6 animals are shown.

Table 3. The effect of 10 μM 5'-AdTdr on the TK1 activity of non-irradiated cells *in vitro* at different concentrations of dTdr in the culture medium. TK1 activity was measured at substrate concentrations of 5 and 60 μM dTdr. Mean values $\pm$ SE of three experiments are given

Conc. dTdr in assay (μM)	TK activity (dpm/ 10^3 S + G ₂ cells/min) dTdr in medium (μM)				
	– 5'-AdTdr			+ 5'-AdTdr	
	0	30	60	30	60
5	172 $\pm$ 5	118 $\pm$ 2	64 $\pm$ 4	222 $\pm$ 2	174 $\pm$ 5
60	349 $\pm$ 6	166 $\pm$ 7	155 $\pm$ 10	383 $\pm$ 2	357 $\pm$ 10

Table 4. The effect of 10 μM 5'-AdTdr on TK1 activity in cells incubated *in vitro* up to 60 min after irradiation *in vivo* with 5.0 Gy. TK1 activity was measured at dTdr substrate concentrations of 5 and 60 μM . Mean values $\pm$ SE of three experiments are given

Time (min)	dTdr(μM) in assay	TK1 activity (dpm/ 10^3 S + G ₂ cells/min)		
		Non-irradiated cells – 5'-AdTdr	Irradiated cells	
			– 5'-AdTdr	+ 5'-AdTdr
30	5	111 $\pm$ 10	67 $\pm$ 3	128 $\pm$ 6
	60	379 $\pm$ 13	170 $\pm$ 8	308 $\pm$ 15
60	5	106 $\pm$ 5	55 $\pm$ 3	112 $\pm$ 8
	60	365 $\pm$ 14	204 $\pm$ 13	336 $\pm$ 9

AdTdr. In the absence of 5'-AdTdr, TK1 activity decreased by about 50%. 5'-AdTdr restored TK1 activity almost completely (Table 4).

Dependence of NaHCO₃ for maintaining TK1 activity in irradiated cells

The dTdr kinetics of TK1 activity was also measured in the presence or absence of NaHCO₃ (1300 mg/l) in the lysing buffer at different concentrations of the substrate (dTdr), 1 and 3 h after irradiation. The linear Michaelis-Menten kinetics of TK1 found in non-irradiated cells changed to biphasic kinetics both in the presence and in the absence of NaHCO₃ after irradiation. However, the degree of inhibition of TK1 activity in the absence of NaHCO₃ was not as

Figure 6. Hofstee plot of substrate (dTdR) kinetics of TK1 in non-irradiated cells (■) and cells at 1 h (▲) and at 3 h (●) after *in situ* irradiation with a dose of 5.0 Gy in the presence (solid symbol) or absence (open symbol) of NaHCO_3 . The inset shows TK1 activity at $2 \mu\text{M}$ dTdR as substrate. TK1 activity is expressed as $\text{dpm}/10^3 \text{ S} + \text{G}_2 \text{ cells}/\text{min}$. Mean values of four mice.

pronounced as in its presence (Fig. 6). Furthermore, at 3 h in the absence of NaHCO_3 , the biphasic kinetics were found only at higher substrate concentrations (5–60 μM dTdR); below 5 μM the kinetics were more or less linear and closely resembled those of the non-irradiated controls cells. This is shown more clearly by the insert in Fig. 6.

DISCUSSION

The dTdR substrate kinetics study of TK1 was performed in crude cell extracts as described in our previous investigation (He *et al.* 1990). Neither dTdR nor dTTP originating from the intact cells significantly affected the determination of the TK1 kinetics. In the present paper we examined the effect of radiation on the dTdR substrate kinetics of TK1 and compared it with the kinetics of the TK1 in non-irradiated cells, and also determined the effect of dTTP on dTdR substrate kinetics in the presence or absence of dTTP of non-irradiated cells. If the decline in TK1 activity after irradiation is due to an elevated intracellular concentration of dTTP, similar dTdR kinetics of TK1 to that found in the non-irradiated cell extract containing dTTP should be observed. According to the Michaelis-Menten constant (K_m) and Hill constant of TK1 in irradiated cells, this was actually the situation over the whole time course up to 24 h, with negative co-operativity being exhibited. The effect of dTTP on dTdR kinetics was previously found in a purified TK1 as described by Munch-Petersen *et al.* (1977, 1984). These results support the hypothesis that the irradiation-induced inhibited TK1 activity is due to feedback regulation of dTTP. The change to a biphasic pattern in the Hofstee plot can be explained by alteration of the substrate affinity to TK1 caused by dTTP.

To demonstrate further the existence of a causal relationship between TK1 activity and dTTP in irradiated cells we used 5'-AdTdR, a dTdR analogue, which is able to eliminate the inhibitory effect of dTTP on TK1 activity in a certain concentration range. This has been demonstrated in intact cells, in cell extracts and in the purified TK1 (Fischer *et al.* 1985). A possible schematic model for the effect of interaction of dTdR, dTTP and 5'-AdTdR on TK1 can be discussed. TK1 has not only catalytic sites for binding of substrate, but also allosteric sites that bind regulatory metabolites such as dTTP. When the allosteric sites are occupied by dTTP, conformational changes of the enzyme appear, leading to inactivation of the catalytic site. When 5'-AdTdR at a lower concentration binds to the catalytic site of the TK1, especially at lower concentrations of the substrate (dTdR) in the TK1 assay, the inhibitory effect of dTTP on the enzyme activity disappears, leading to almost normal enzyme activity. 5'-AdTdR completely reversed the irradiation-induced inhibition of TK1 activity and, thus, strongly supports the assumption that the inhibition of TK1 activity after irradiation is connected to changes of dTTP.

Earlier studies found that the reduced TK1 activity after irradiation was maintained only in a certain range of pH (7.0–7.2) and NaHCO_3 concentration (1200–1400 mg/l) (Feinendegen *et al.* 1988). It was therefore of considerable interest to investigate further the influence of NaHCO_3 on TK1 activity. We found, depending on the substrate concentration used in the TK1 assay *in vitro* and the time after irradiation, an inhibition of TK1 activity also without NaHCO_3 . Thus, soon after irradiation, NaHCO_3 was not essential to maintain the irradiation-induced inhibited TK1 activity if substrate concentrations were used corresponding to those of intact cells. At later times, however, the presence of NaHCO_3 was needed. An important conclusion from this finding is that the inhibition of TK1 activity is not an artifact due to manipulation of the compounds of the lysing buffer. The mechanism(s) of action of NaHCO_3 is still unclear. It has been suggested that NaHCO_3 is needed to control the intracellular pH, which is necessary to elicit the effect of irradiation on TK1 activity (Feinendegen *et al.* 1984, 1988). This may imply that NaHCO_3 acts as an analogue of intracellular ions to maintain the concentration and the equilibrium of ions in the TK1 assay, since the ions from the cells are greatly diluted. Regarding the elevated cellular concentration of dTTP for the irradiation-induced inhibition of TK1 activity, it could be argued that an optimal balance of ions is necessary to prevent the release of dTTP from the inhibitory site of the enzyme. However, the independence of the inhibition of TK1 activity of NaHCO_3 early after irradiation, and the close correlation between TK1 activity and concentration of dTTP in non-irradiated cells, despite the lack of NaHCO_3 in the lysing buffer, make such a suggestion less likely.

Another important observation was that in the presence of NaHCO_3 TK1 activity was constantly inhibited with time at a low substrate concentration (2–3 μM), while TK1 activity fluctuated at a saturated substrate concentration (60 μM), as shown in paper I. However, in the absence of NaHCO_3 and at a concentration of 2 μM dTdR, which is found in the intact cell, only a transient decrease in TK1 activity 1 h after irradiation could be observed.

In conclusion, dTTP plays a major role in the radiation-induced inhibition of TK1 activity. The role of NaHCO_3 in maintaining the inhibition of TK1 activity is still unclear.

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