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Bone cancer risk in mice exposed to ^{224}Ra: protraction effects from promotion

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Abstract This paper analyzes data for the osteosarcoma incidence in life-time experiments of ^{224}Ra injected mice with respect to the importance of initiating and promoting action of ionizing high LET-radiation. This was done with the biologically motivated two step clonal expansion (TSCE) model of tumor induction. Experimentally derived osteosarcoma incidence in 1,194 mice following exposure to ^{224}Ra with different total radiation doses and different fractionation patterns were analyzed together with incidence data from 1,710 unirradiated control animals. Effects of radiation on the initiating event and on the clonal expansion rate, i.e. on promotion were found to be necessary to explain the observed patterns with this model. The data show a distinct inverse protraction effect at high doses, whereas at lower doses this effect becomes insignificant. Such a behavior is well reproduced in the proposed model: At dose rates above 6 mGy/day a longer exposure produces higher ERR per dose, while for lower rates the reverse is the case. The TSCE model permits the deduction of several kinetic parameters of a postulated two-step bone tumorigenesis process. Mean exposure rates of 0.13 mGy/day are found to double the baseline initiation rate. At rates above 100 mGy/day, the initiation rate decreases. The clonal expansion rate is doubled at 8 mGy/day, and it levels out at rates beyond 100 mGy/day.

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Introduction

After its discovery in 1898 by Marie and Pierre Curie, the long-lived radio-nuclide ^{226}Ra (half life 1,620 years) found various medical and technical applications. Excess osteosarcomas were detected in dial painters who had incorporated ^{226}Ra from fluorescent paint already as early as the late 1920s and early 1930s. There exist many epidemiological studies on this subject. A review of those conducted in the USA is given by Rowland [1]. The observations of adverse health effects in humans triggered several animal experiments aiming at a better understanding of the mechanisms of induction of osteosarcomas by incorporated radium. For this reason at the GSF Research Center in Neuherberg, Germany, mice were exposed to various bone-seeking radionuclides (mainly to the short-lived ^{224}Ra, with a half life of 3.66 days) [2, 3, 4]. The work is still ongoing, with a recent shift in emphasis to identify the genes involved in osteosarcoma predisposition [5, 6]. The early experiments with high total doses of ^{224}Ra showed distinct inverse protraction effects: When the same total activity was administered by repeated injections over a longer period, the osteosarcoma frequency increased significantly and the latency period became shorter [2]. This effect was more pronounced at higher exposure values and decreased or, possibly, even vanished at the lowest injected activity of 0.5 μCi/kg (i.e.1.85 $\times$ 10^4 Bq/kg) [3]. [1]

A similar protraction behavior has been observed in fatal lung tumors in rats induced by radon [7]. This pattern could be explained by a two stage clonal expansion (TSCE) model (Fig.1)[8, 9, 10] which can be thought of as a mathematical formalization of the initiation-promotion-progression paradigm of carcinogenesis: Radon has been found to act on both, the initiation and promotion (clonal expansion) during lung tumori-

[1]These experiments were mainly performed before the official introduction of SI-units in Germany in 1985 (1μCi/kg $\equiv$ 3.7 $\times$ 10^4 Bq)

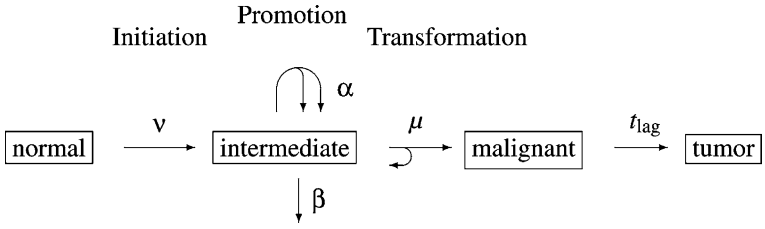

Fig. 1 Sketch of the TSCE model

genesis, with action on promotion being responsible for the inverse protraction effect observed at high exposures. Thus the question arose, whether such a mechanism could also explain the observed pattern in bone sarcoma induction by radium incorporation.

Some of the features of the TSCE model, like initiation and promotion are similar to what was assumed in the model of Marshall and Groer [11]. Related models were also applied to the data of radium-induced osteosarcomas in beagles [12, 13, 14], but in these papers a promoting action of radiation was not tested.

In the present study we analyzed data on osteosarcoma induction after exposure to ^{224}Ra, as this isotope has a short physical half-life of 3.66 days and therefore allows a reliable determination of the exposure duration in fractionation experiments. The aim is to study the radiation action in osteosarcoma induction within the TSCE-model, and to estimate kinetic parameters with a potential biological meaning. The renaissance of the treatment of ankylosing spondylitis with ^{224}Ra chloride [15, 16] gives further motivation to study the risk of osteosarcoma induction at therapeutic doses.

Materials and methods

Data set

A data set of 2,904 female NMRI mice was selected; 1,710 of these mice were controls with 13 osteosarcoma cases. The other 1,194 mice were exposed to various fractionation patterns of ^{224}Ra. Of these 301 finally developed at least one bone tumor. Table 1 gives the number of animals and the number of those with osteosarcoma in each group. The groups with 4 μCi/kg exposure or more are presented in Ref. [2]. The groups with an exposure of 0.5 μCi/kg are discussed in Ref. [3]. The controls are the standard ones collected over a long period at the Institute for Pathology of the GSF research center, Neuherberg.

For each mouse the start and end of the follow-up is taken as well as the status on bone tumor (yes or no) and the age-dependence of exposure, as discussed below. The follow-up for all mice began at 30 days of age; this was also the start of exposure. For the control animals, only the number of mice, the age at death for the osteosarcoma cases, and the number of mice alive at these dates (this is the information necessary for Kaplan-Meier plots) were known. Therefore, the lifetime of the mice which died between two of these ages is distributed evenly in the interval given by the two ages. For the mice which died before the first occurrence of an osteosarcoma, 200 days is taken as the lower end of the interval, and for the mice which were still alive when the last osteosarcoma occured, 1,000 days is taken as the upper end of the interval.

^{224}Ra was injected into mice in the form of radium-chloride. If there were more than one injection, they were applied at intervals of 3.5 days. The number of injections and the injected activity in μCi/kg are given in

Table 1 Groups of female NMRI mice used in this analysis. The total exposure and the fractionation scheme is given. The interval between injections is 3.5 days. Two groups with identical exposure pattern are combined. The expected number of cases and of baseline cases is calculated with the preferred model

Group(s)	Exposure (μCi/kg)		No. of mice	No. of cases	No. of cases	
	Total	Fraction			expected	baseline
Controls	0		1710	13	15.4	15.4
RGA-0.5	0.5	1×0.5	249	16	11.4	1.75
RKC-0.5	0.5	72×0.007	299	20	11.5	2.74
RKG-4	4	1×4	75	10	13.7	0.56
RKC-4	4	72×0.056	74	10	19.8	0.50
RKB-12	12	1×12	49	11	9.3	0.34
RKA-12	12	8×1.5	50	18	19.9	0.24
RK-12	12	24×0.5	49	23	23.1	0.12
RKB-36 +RKE-36	36	1×36	100	13	11.5	0.42
RKA-36	36	8×4.5	50	10	9.1	0.15
RK-36	36	24×1.5	50	30	28.7	0.07
RKC-36	36	72×0.5	99	96	93.8	0.05
RKD-36	75	50×1.5	50	44	46.8	0.02
Total			2904	315	314.0	22.32

Table 1. From this information the age-dependence of the dose rate to the bone is calculated. Briefly, the half-life of ^{224}Ra of 3.66 days reduces the activity to 0.5154 after 3.5 days. In the first interval of 3.5 days the nominal injected quantity is used as the nominal exposure rate, and in each subsequent interval of 3.5 days, a quantity reduced by that factor. Fourteen reduction intervals are considered, thus decreasing the exposure rate by up to a factor of about 10,000. The largest injection of 36 μCi/kg is reduced to about 0.0036, i.e. to a smaller activity than the smallest injected one. The resulting exposure rates are plotted in Fig.2 for each of the exposed groups. This substitute for the dose rate to the bone is used as it is a well determined quantity and gives a good approximation to the true time-dependence of the dose. A conversion to dose rate in mGy/day is given in the discussion section together with remarks on the uncertainties of such a number. The precise age dependence is a zig-zag curve shown for example in Ref. [2, Fig.2]. The approximation used here describes the protraction patterns of the experiment very well, but averages over the age dependence in the 3.5 day intervals.

The mathematical model

The mathematical model applied here is a two step carcinogenesis model which accounts for clonal expansion of initiated cells, and also allows effects of radiation on this (TSCE-model). This model has a long history; short introductions and references can be found in [17, 18, 19]. A sketch of the model is given in Fig.1. Intermediate cells are created from normal cells with the initiation rate ν which has a baseline and a radiation-related part. Intermediate cells can divide into two intermediate cells with a rate α, die or differentiate with a rate β, and they can divide into an intermediate cell and a malignant cell with a transformation rate μ.[2] The effective clonal expansion rate is $\alpha-\beta-\mu$. The progression from a malignant cell to an observable tumor is described in the model by a lag time t_{lag}. Not all of these parameters can be determined from an analysis of the data [20, 21]. Therefore, only identifiable parameters are used here. The exact hazard function for the stochastic TSCE model with constant parameters has been published frequently e.g. [19] and is therefore not repeated. The recursive algorithm described in Ref. [21] is used to calculate the hazard and the probability of cancer for parameters which are piecewise constant in age.

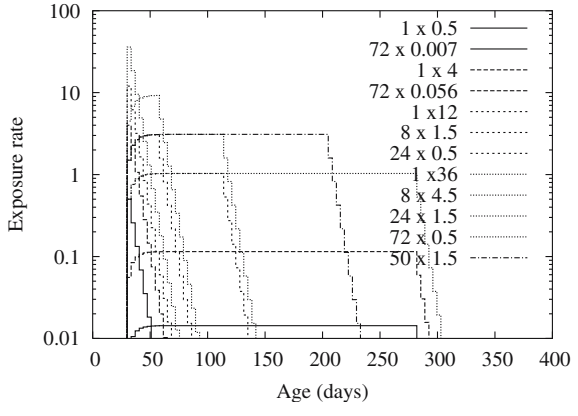

Fig. 2 Age-dependence of exposure rate, as used in this paper. As described in the text, the unit of d is the exposure rate after an injection of 1 μCi/kg ^{224}Ra, averaged over 3.5 days. Fractionation patterns with the same total exposure are plotted with the same line style, as they can be separated by the different durations

The original version of the model assumed that the initiation rate and transformation rate describe two mutations e.g. of two alleles of a tumor-suppressor gene. However, the mathematics of the model can describe a much wider range of biological processes, which includes more than one event, to obtain clonal advantage, bystander effects, and genomic instability, even as an epigenetic event. In this view the parameter values estimated from cancer data sets describe effective processes which can in principle be compared with molecular biological experiments [19]. The model parameters used here are selected with such an aim in mind.

The model allows different dependencies of the parameters on exposure rate. Radiation action on initiation, the effective clonal expansion rate (promotion), and transformation are here assumed to be possible in principle. These different radiation actions can be quantified by fitting this versatile model to data that are sufficiently powerful statistically. The dependency on exposure rate which is used is described next.

The total initiation rate at the dose rate d divided by the baseline initiation rate (i.e. without artificial irradiation of the animal) is assumed to be of the general form

$$\frac{\nu(d)}{\nu(0)} = 1 + \nu_1 d \mathrm{e}^{-\nu_2 d}. \tag{1}$$

The parameter ν_1 can be interpreted as the inverse of the dose rate which doubles the initiation rate. And ν_2 is a term which traditionally is assumed to be related to cell killing (which is usually exponential for high-LET irradiation). The effective clonal expansion rate $\gamma(d) \equiv \alpha(d)-\beta(d)-\mu(d)$ in this model is assumed to be dependent on the dose rate such that it is linear with coefficient γ_{lin} at low rates, and levels to a value of $\gamma_0+\gamma_{\mathrm{level}}$ at high dose rates:

$$\gamma(d) = \gamma_0 + (1 - \mathrm{e}^{-(\gamma_{lin}/\gamma_{level})d}). \tag{2}$$

For the transformation rate divided by the baseline one, a linear dependence on dose rate is assumed in this model:

$$\frac{\mu(d)}{\mu(0)} = 1 + \mu_1 d. \tag{3}$$

[2]The term transformation is used here for the rate limiting event from an intermediate cell to a malignant one, **not** for the whole process from a healthy to a malignant cell.

The six parameters in the equations above are estimated in addition to three parameters which affect the baseline rates. One of the latter is the product $Y_0 \equiv \nu(0)\mu(0)$ of the baseline initiation and transformation rates. Another is the parameter q, which leads to a levelling, at high age, of the hazard at $Y(0)/q$. For small μ it is approximately $q \approx \mu/(1-\beta/\alpha)$ [21]. The lag time t_{lag} is also estimated from the data.

Likelihoods and quality of fit

The mathematical formulation of the TSCE model allows to calculate the hazard and the respective "survival" probability S (i.e. the probability that no osteosarcoma has occurred) at the age of death. The time at risk from the beginning of the follow-up period is used. Model parameter fitting is done by maximizing the log-likelihood

$$\ln L = \sum_{\text{no cancer}} \ln S_i + \sum_{\text{cancer}} \ln (h_i S_i). \quad (4)$$

This form assumes that all osteosarcomas are fatal. The deviance is defined as

$$\text{Dev} = -2 \ln L_{\max} \quad (5)$$

for the maximum likelihood. Parameter uncertainties are calculated using the profile likelihood technique (MINOS in the software package MINUIT from CERN [22]).

As a means for judging the quality of fits, the Kaplan-Meier estimate of the probability of tumor is compared with that from the fitted model, separately for each experimental group. Also for each group the number of expected cases is calculated by summing over all animals the cumulative hazard for each animal during the follow-up.

Results

The results for the deviance of some of the model fits are given in Table 2. The full model is the one described above, with nine parameters. Inspection of the estimated errors of the parameters indicate that two of them do not differ significantly from zero. When postulating no transforming effect of radiation by fixing $\mu_1 = 0$, the loss in deviance (0.03) is negligible. Also, fixing the parameter q at a very small positive number gives only a minor decrease in the deviance. This means that the data do not show a levelling of risk at the age reached by the mice. Based on these observations the model with seven parameters was preferred. Its estimated parameter values are given in Table 3 together with their standard error confidence bounds. Models with either an initiating or a promoting action of radiation turned off were also fitted; the deviance increases substantially in these cases (Table 2).

In Fig. 3, the Kaplan-Meier estimates of the probability of tumor incidence and those expected from the

Table 2 Comparison of various models showing their deviance, and the number of fitted parameters

Model	Deviance	No. of parameters
Full	4462.667	9
No transformation ($\mu_1 = 0$)	4462.700	8
Preferred ($q = 0$, $\mu_1 = 0$)	4462.731	7
No promotion	4655.079	5
No initiation	4743.027	5

Table 3 Maximum likelihood estimates (MLE) for the parameters of the preferred model, and their standard error confidence bounds based on profile likelihood calculations. As described in the text, the unit of d is the exposure rate after an injection of 1 μCi/kg averaged over 3.5 days

Parameter	MLE	Conf. bound
Y_0 (day^{-2})	0.62×10^{-8}	(0.43, 0.90)
ν_1 (d^{-1})	311	(216, 452)
ν_2 (d^{-1})	0.38	(0.31, 0.46)
γ_0 (day^{-1})	0.61×10^{-2}	(0.57, 0.66)
γ_{lin} ($(d\ \text{day})^{-1}$)	0.030	(0.026, 0.035)
γ_{level} (day^{-1})	0.0208	(0.0191, 0.0226)
t_{lag} (day)	72.5	(62.4, 81.7)

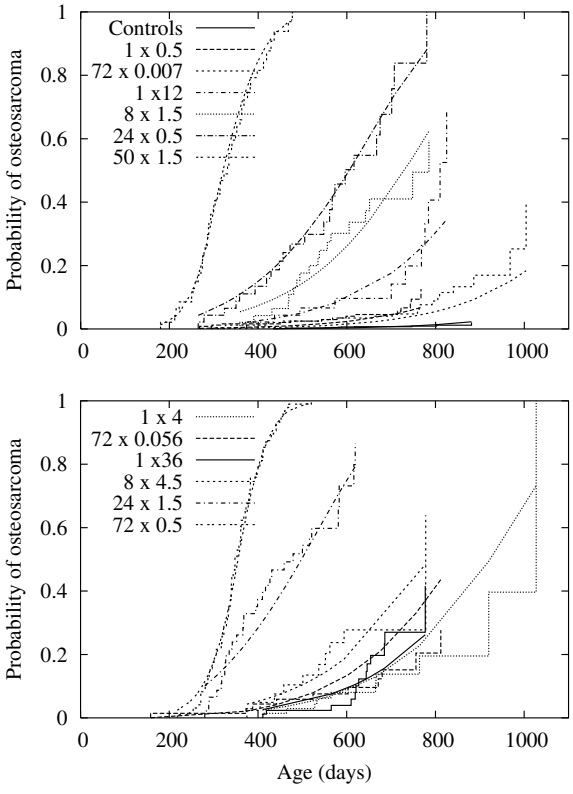

Fig. 3 Probability of osteosarcoma incidence for each experimental group from the Kaplan-Meier estimate (*steps*) and calculated with the preferred TSCE model (*smooth lines*). The groups are sorted by increasing total exposure, and then by longer protraction. This gives roughly a sorting from late effect to earlier effect

preferred TSCE model are plotted. In general, the data are described satisfactorily. In Table 1 the number of cases expected with the model is given, and also the number expected from the model for baseline tumors. The hazard functions and derived quantities like the relative risk function can be calculated from the estimated parameters. As an example the estimated relative risk functions are plotted in Fig. 4.

Discussion

The proposed model can well describe the observed protraction effects for the same total exposure. Also, the effects of protraction at the same dose rate are described satisfactorily: See in Fig.3 the groups with injections of 0.5 μCi/kg (1×0.5, 24×0.5 and 72×0.5) and the groups with injections of 1.5 μCi/kg (8×1.5, 24×1.5 and 50×1.5). In such a comparison, the dose rate dependent expressions $\nu(d)$, $\gamma(d)$ remain essentially unchanged between the different exposure patterns so that only the different exposure periods are left in the model to predict different outcomes, see Fig.2. Therefore, this type of analysis is particularly reliable for testing the promoting action of radiation [23].

For the two groups with a total exposure of 0.5 μCi/kg (i.e. single 0.5 respectively 72×0.007 μCi/kg) the model predicts a smaller number of cases than was observed. These experiments were done several years after the others. Moreover, the control group had only 99 animals in this experimental series with three tumors [3] whereas less than one would have been expected from the other control animals. This may point to a higher risk of osteosarcoma in the non-exposed animals used at that time, possibly due to genetic, epigenetic, environmental or other not quantified factors. For the group RKC-4 the model predicts too many cases. This may be due to the groups mentioned above which enhance the estimated risk. But even mere statistical fluctuations could explain this stray result.

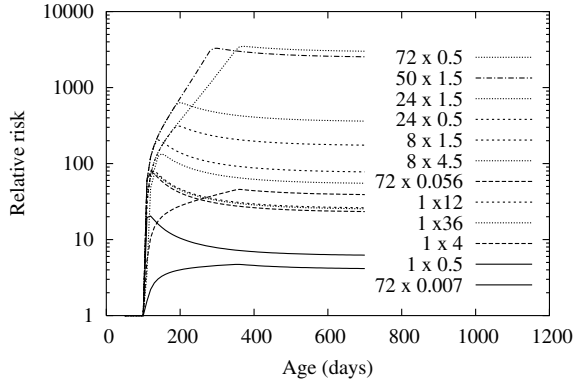

Fig. 4 Relative risk functions for each exposure group as calculated with the preferred model. The groups in the legend are sorted by the asymptotic relative risk. Groups with the same total exposure have the same line style

Initiation and promotion are both needed to interpret the data, as can be seen from the deviances in Table 2. This is in contrast to the assumptions made in earlier papers with osteosarcoma induction in beagles [12, 13, 14]. Promotion action of radiation alone can describe the protraction effects at 36 μCi/kg quite well. Initiation alone can describe the short exposures. But each of them individually fails to describe the exposure patterns consistently.

Radiation induced and baseline osteosarcomas occur preferentially at the same anatomical sites of the skeleton [4]. This can be understood by the present model where the initiated intermediate cells are promoted proportionally [19].

So far, the exposure has been described using the injected amount of ^{224}Ra in order to avoid uncertainties in the estimation of dose to the target zone. For the discussion a conversion to dose is useful. In the two papers [2, 3] the estimated mean skeletal dose after an injection of 1 μCi/kg is calculated as 300 mGy. This allows converting the scale for exposure rate used above to a skeletal dose rate of 41.5 mGy/day, or about 40 mGy/day. This conversion factor is used here.

The local bone dose may vary considerably. The highest doses occur at sites of high calcium metabolism at the time of incorporation e.g. at the so-called bone surface. The dose rates in these areas may be higher than the mean skeletal values by a factor of 10 or even higher [2]. The cells at risk, however, lie possibly not in the most irradiated zone (see below). Such factors are restricted here to the conversion coefficient and can be directly taken into account when desired.

The estimated dependence of initiation and promotion on dose rate is shown in Fig. 5. The preferred model for osteosarcoma incidence in mice can now be compared with the model for fatal lung tumor in rats [9], when a conversion of WLM in organ dose in rats is known. A value of 7.4 mGy WLM^{-1} is used [24]. In both cases of mainly alpha-particle irradiation no significant transforming action of radiation was found but only an action on initiation and on promotion.

For osteosarcoma initiation the doubling dose rate is about 0.13 mGy/day. This is lower than the 3 mGy/day for lung tumor induction. The initiation increases with dose rate up to 100 mGy/day maximum, then declines at even higher dose rates. This effect is caused by the groups with single injections: exposures higher than about 4 μCi/kg have only small additional effect. The preferred model can describe this only by giving little effect to the high dose rates. The true reason for this saturation may be of a different nature. Possibly the history of exposures needs to be taken into account. No decrease in initiation rate was found in fatal lung tumors up to the maximum dose rate of 500 mGy/day.

The levelling of the effective clonal expansion rate is caused by the groups with high exposure rate over a long time. It is somewhat surprising that the initiation parameter peaks at about the same dose-rate as when the promotion parameter reaches its plateau, given that

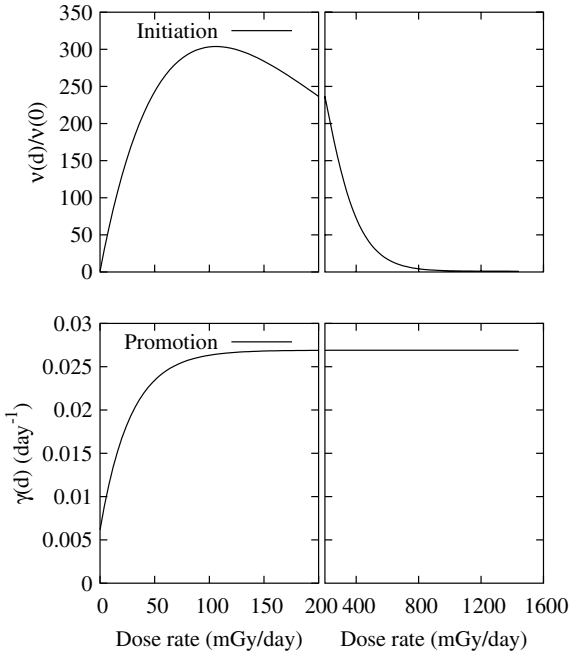

Fig. 5 Dependence of initiation and promotion parameters on dose rate d

these two phenomena are caused by different features of the data set (short exposure times versus long exposure times). The promoting effect in the osteosarcoma carcinogenesis process is estimated to increase the effective clonal expansion rate by a factor of about 4.2. In fatal lung tumors in rats, this increase was by a factor of 3.8. The growth rate of intermediate cells doubles in the mice at about 8 mGy/day and in the rats at about 35 mGy/day.

The most prominent feature of the osteosarcoma induction at higher doses is the observed massive inverse protraction effect. In the context of the TSCE-model used here this could be explained by a promoting effect of radiation. Fig. 6 shows the calculated excess relative risk at an age of 700 days for various dose rates and exposure durations typical for the experiment. As can be seen, at dose rates above 6 mGy/day a longer exposure produces higher ERR per dose, while for lower rates the reverse is the case. Note that the inverse protraction effect starts well below the dose rate where the promotion parameter reaches its plateau. It is a consequence of the relative importance of initiation and promotion, not of the levelling of the promoting effect at high dose rates. The usual protraction effect below 6 mGy/day is mainly due to an age-at-exposure effect. For dose rates around 6 mGy/day, protraction has little effect on the ERR per dose. This situation again is very similar to what was found for lung tumor induction by radon in rats, see [9, Fig. 6]. The turnover between the two protraction effects in lung tumor induction was found to be at 15 WL, or about 16 mGy/day.

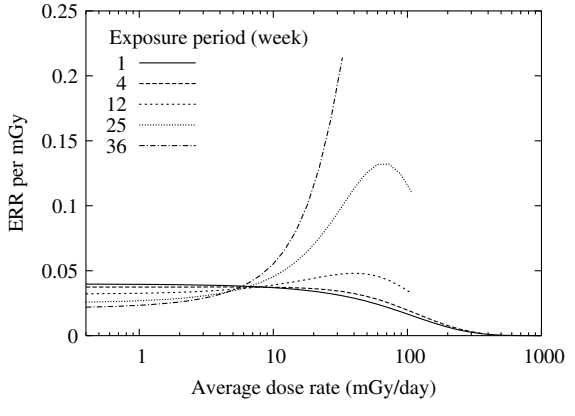

Fig. 6 The calculated excess relative risk per dose at an age of 700 days, for exposure from an age of 30 days for the period (in weeks) given in the key. The *curves* are limited to dose rates actually used in the experiment

So far the analysis has estimated the kinetic parameters of the process of osteosarcoma induction by radium from the data. These kinetic parameters should be compared with parameter estimates from other sources. As a step in that direction some simplified and highly uncertain microdosimetric speculations are presented.

A possible promoting effect of radiation in the induction of lung tumour has been related to the replacement of inactivated cells [25]. One of us (M.R.) has measured radiation-induced cell inactivation following gamma-exposure in a culture of rat osteoblasts (ROS17/2.8) with a standard assay. They exhibit a pure exponential survival curve

$$S = e^{-\alpha D} \tag{6}$$

with $\alpha = 1.4 \pm 0.2$ Gy^{-1}. The use of an RBE of alpha particles of about 2 for this endpoint [26], implies that 1 μCi/kg injected ^{224}Ra (equivalent to 0.3 Gy of alpha dose) would inactivate about 43% of all the cells within the range of the alpha-particle, which is about 45 μm [27]. Differentiated osteoblasts are located in the so-called osteoid seam which extends beyond the mineralization front and has a thickness between 5 μm and 50 μm [28]. This would imply that a sizable fraction of all differentiated osteoblasts are killed at the lower exposures rates, and virtually all at the higher exposure rates. But these cells are not likely candidates as precursor cells of osteosarcomas as they do not divide. The pre-osteoblasts are partly beyond the range of the alpha-radiation, but have a limited proliferation capacity [29]. This and further histopathological observations suggest that the best candidates for the cells at risk are multipotent and totipotent mesenchymal precursors located at a distance of several tens of μm beyond 10 μm [30]. The cells at risk in the TSCE model must satisfy several conditions: They must essentially maintain the changes towards cancer cells for their full life and they must be exposed to ionization events (or influenced by bystander effects). The intermediate cells must undergo a slow clonal advantage (about 0.6% per day) which requires a

symmetric division of these cells. The candidate cells mentioned above satisfy these conditions. If the cells can undergo the initiating event(s) due to ionization, then neighboring cells can also be inactivated, and the cell replacement mechanism could be a reason for the radiation induced promotion. It is hoped that the recent work on genetic predisposition for osteosarcoma in mice [5, 6] will lead to an identification of the genes (or groups of genes) involved in the early events of mouse osteosarcomas.

The two-compartment approach used here avoids the problems of extensive cell killing of the bone tumor target cells as brought forward by Marshall and Groer [11]. We used the idea that differentiated osteoblasts, which are located in direct proximity to the ^{224}Ra deposits, are preferentially killed and lead to an accelerated repopulation by the multipotent mesenchymal stem cells by asymmetric cell division. This asymmetric division does not change the number of intermediate cells. Only symmetric division of initiated mesenchymal stem cells can give the clonal advantage. As compared to the traditional concept of autonomous and uniform cell entities progressing from a normal to complete malignant state, the postulated two compartment tissue system is closer to the real architecture of the bone.

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