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DOSE- AND TIME-DEPENDENT INCREASE OF LYSOSOMAL ENZYMES IN EMBRYONIC CARTILAGE IN VITRO AFTER IONIZING RADIATION

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Abstract

Radiation doses of 20, 50 or 100 Gy caused the same time related decrease for RNA and proteoglycan (PG) synthesis in embryonic cartilage in vitro (4 days culture). In this paper, participation of lysosomes in this radiation response is investigated. Therefore, we employ a cytochemical method using β-glycerophosphate as substrate for acid phosphatase (AP) detection. Increase of AP was found 2 days after irradiation and increased during the whole culture period. The increase was more pronounced with a higher radiation dose. Stimulation of AP activity explains the observed radiation response of RNA and PG synthesis.

KEY WORDS : Cytochemistry, Embryonic cartilage, Ionizing radiation, In vitro, Acid phosphatase, Lysosome, β-glycerophosphate.

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Introduction

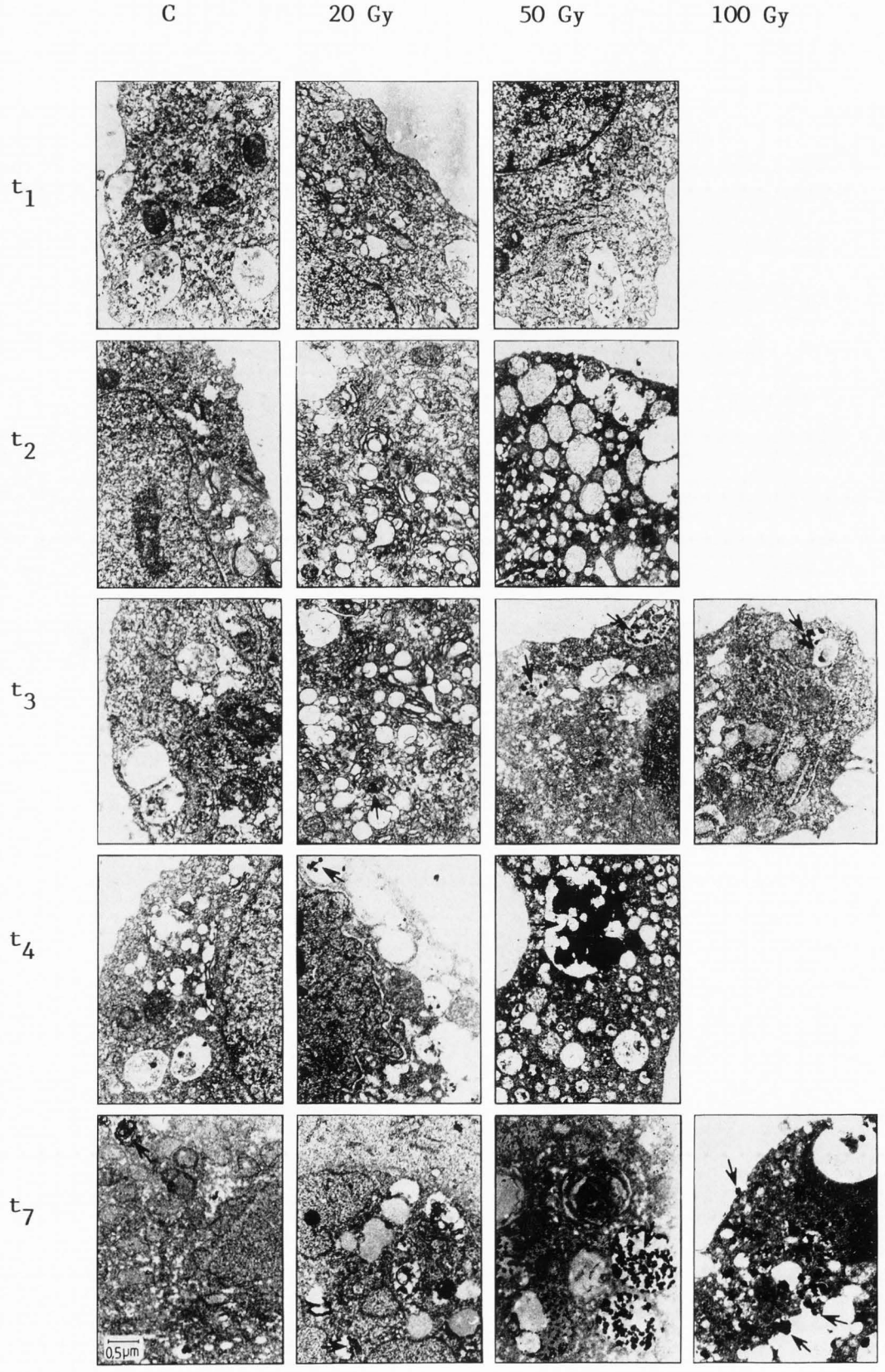
In previous papers (Cornelissen et al., 1990) the radiation response of RNA and PG synthesis in embryonic cartilage in vitro was discussed. Radiation caused a limited inhibition of synthesis, immediately after irradiation. This inhibition remained unaffected during 2 days after irradiation. From then on a more pronounced effect was found which increased during the whole culture period. For each radiation dose (20, 50, 100 Gy) the same time-related pattern of synthesis inhibition was found. Since ionizing radiation can increase the activity of certain enzymes, and since lysosomal enzymes are known to affect cell metabolism (Bacq and Alexander, 1961; Prasad, 1984), we want to find out if a correlation exists between lysosomal activity and the observed radiation response.

Materials and methods

Isolation procedure. Tibiae from 6.5 day old chick embryos (stage 30, Hamburger and Hamilton, 1951) were mechanically freed from the surrounding connective tissue by rolling them on sterile filter paper. Of each embryo, one tibia was irradiated, the counterpart served as sham-irradiated control.

Irradiation technique. Irradiation was performed with a Philips MG 420 X-ray system, operating at 250 kV constant potential with an additional filtration of 1 mm Al and 1.1 mm Cu, corresponding to a radiation quality with a measured HVL of 2.1 mm Cu. Tibiae were lying in plastic vials containing sterile Ringer solution and placed in a holder, submerged in a water phantom. During irradiation the tibiae as well as the counterparts were kept at 37°C. The tibiae were exposed bilaterally at a dose rate of 1.5 Gy/min. Single radiation doses of 20, 50 or 100 Gy were used. Estimation of these doses was obtained by placing a dosimeter for absorbed dose at the place of the specimen.

Culture technique. After irradiation, irradiated and control tibiae were cultured for 1, 2, 3, 4 or 7 days in liquid Eagle's Minimum Essential Medium with Hanks' salts, supplemented with 10% foetal calf serum and antibiotics. Morphological alterations of the tibiae related


C
20 Gy
50 Gy
100 Gy
t1
t2
t3
t4
t7
0,5µm

to radiation dose and culture conditions are described in a previous paper (de Ridder et al., 1988).

Cytochemical preparation. After culturing, tibiae were prepared for the ultrastructural demonstration of acid phosphatase (AP), following the method of Miller and Palade (1964). This method is a modification of the Gomori Method, applied in light microscopy. For each radiation dose and for each post-irradiation time at least 3 specimen were considered. Tibial epiphyses were fixed for 1 hour in 2.5% glutaraldehyde buffered in 0.1 M cacodylate buffer. After fixation, they were washed in cacodylate buffer with 7.5% sucrose. Afterwards, either small pieces or frozen sections (30 µ) were incubated for 45 minutes at 37°C at pH 5.1 in a medium containing 3% Gomori β-glycerophosphate and 0.12% lead nitrate. Presence of AP results in the formation of an electron dense lead phosphate precipitate. However, incubation of small pieces gave non-reliable results: in the same tissue a great variation in Pb phosphate precipitates was found. This speaks for an incomplete penetration of the incubation medium. Therefore the following results only concern observations made on frozen sections. As a control of the incubation method, sections were incubated in the same medium without β-glycerophosphate. After incubation, the sections were washed in buffer, post-fixed in osmium tetroxide, embedded in ERL and cut in 70 nm sections. Stained (uranyl acetate and lead citrate) and unstained sections were examined under a Jeol 1200 EX/II electron microscope.

Results

Results of electron microscopic investigations, made on frozen sections, are given in Table I. This table presents the number of cells containing AP to a greater or smaller extent. The number of AP positive cells as a function of radiation dose and of post-irradiation time is given. For each value, 100 cells were counted. For a radiation dose of 100 Gy only 2 time points (t_3 and t_7) were used.

The number of cells with AP activity increases with the time of culturing, even in non-irradiated tibiae. After a culture period of 7 days 20/100 cells show AP activity instead of 4/100 cells at the start of the culture. However, an additional effect, due to irradiation, is observed during the same period. Until 2 days after irradiation, no significant differences between irradiated and control tibiae are found. From then on, irradiated cartilage shows more AP positive cells than non-irradiated cartilage; this number increases as a function of the post-irradiation time.

Figure 1 : Epiphyseal cells of tibiae, cultured for 1, 2, 3, 4 or 7 days (respectively t_1, t_2, t_3, t_4, t_7) and treated for AP demonstrations. Control tibiae (C) and irradiated tibiae (20 Gy, 50 Gy, 100 Gy) are presented. AP activity is visible as electron dense precipitates.

Table I: Number of cells/100 cells with Pb phosphate precipitate.

	C	20 Gy	50 Gy	100 Gy
t_1	4	4	6	
t_2	8	7	9	
t_3	11	24	42	30
t_4	16	28	48	
t_7	20	34	68	54

C = controls
t_1, t_2, t_3, t_4, t_7 respectively 1, 2, 3, 4 or 7 days after irradiation.

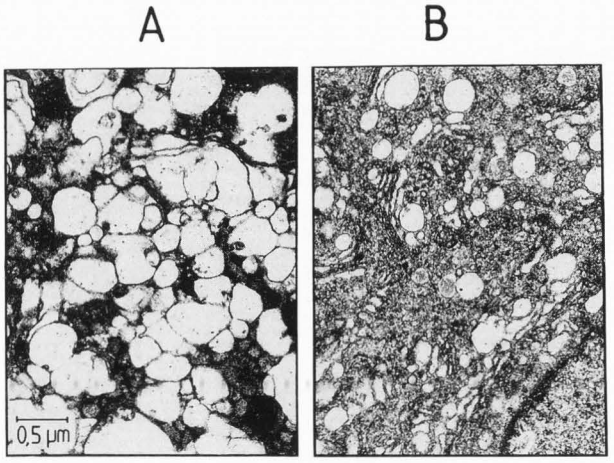


Figure 2 : Epiphyseal cells of tibiae, irradiated with 50 Gy and cultured for 4 (fig. 2b) or 7 days (fig. 2a). Tibiae were incubated in the incubation medium without β-glycerophosphate. No Pb phosphate precipitates are visible.

Not only the number of AP rich cells changes after irradiation: also the amount of precipitate and the ultrastructure in which Pb phosphate could be found show variations.

This is illustrated in fig. 1. For each radiation dose and for each post-irradiation time, a cell, representative for the presence of AP in that particular tissue, is presented. Until 2 days after irradiation most cells are AP negative, as well for the irradiated tibiae as for the controls. From day 3 on, variations in Pb phosphate precipitates, as a function of radiation dose and of post-irradiation time are observed. Control tibiae and tibiae irradiated with 20 Gy show dense precipitates from day 4, those irradiated with 50 or 100 Gy from day 3. In all cases, Pb phosphate precipitates are restricted to vesicles and larger vacuoles, and exceptionally to dilatations of the RER. On day 7, for controls and tibiae irradiated with 20 or 50 Gy, precipitates remain restricted to vesicles and vacuoles. However, for tibiae

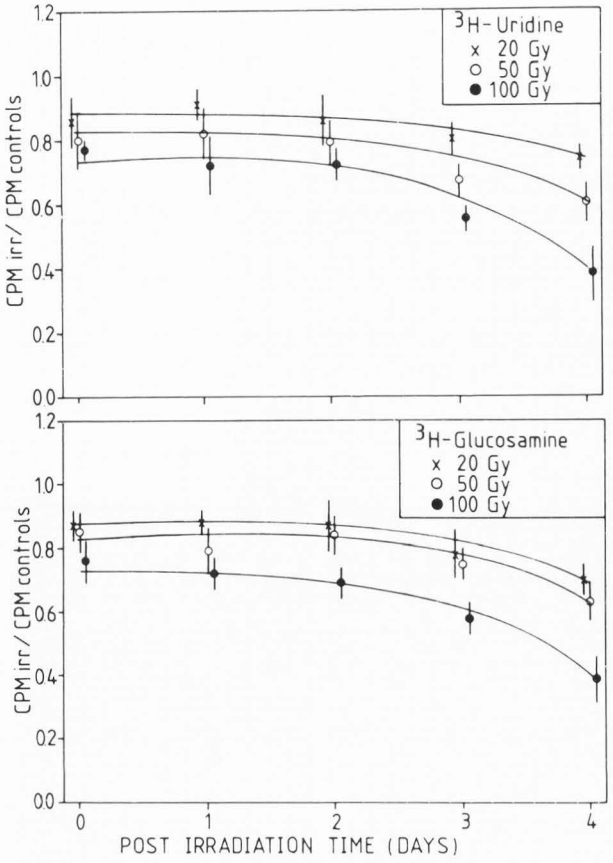


Figure 3: Effect of different radiation doses (20, 50, 100 Gy) on the incorporation of ^{3}H-uridine (fig. 3a) and ^{3}H-glucosamine (fig. 3b) at different times after irradiation.

CPM irr = counts per minute for the irradiated tibiae.

CPM controls = counts per minute for control tibiae (counted by liquid scintillation counting). (From Cornelissen et al., 1990).

irradiated with 100 Gy, precipitates are also spread in the cytoplasm and even in the extracellular matrix.

As a control of the incubation method, tibiae irradiated with 50 Gy and cultured for 4 (fig. 2b) or 7 days (fig. 2a), were incubated on the same incubation medium, without β-glycerophosphate. As illustrated in fig. 2, no Pb phosphate precipitates were found in those cells.

Discussion

From previous papers (Cornelissen et al., 1990) it appeared that RNA synthesis (^{3}H- uridine incorporation) and proteoglycan synthesis (^{3}H-glucosamine incorporation) showed the same time-related radiation response in irradiated embryonic cartilage in vitro. This radiation response is presented in fig. 3 (a, b). Since cartilage tissue is extremely radioresistant and since we were interested in acute effects related to metabolic disturbances (RNA and GAG synthesis) doses higher than 20 Gy were required.

Briefly : for each radiation dose (20, 50, 100 Gy) the inhibition remained at the same level during the first two days after irradiation. Dependent on the radiation dose a limited immediate effect was observed (= initial inhibition), although higher with higher doses. From then on and for each radiation dose, a decrease in synthesis was found. This decrease continued during the whole culture period (4 days). The observed radiation pattern speaks for :

1. either a pool of enzymes, already present at the moment of irradiation and still functional for a given time after irradiation

2. either synthesis or activation of enzymes deleting cells or lowering the metabolic activity of the cells.

In the above mentioned paper it was observed that a pool of enzymes (checked for RNA polymerase concerning RNA synthesis) could not explain the observed radiation pattern.

Activation of certain enzymes after irradiation is already mentioned by Kelly (1961) and Bacq and Alexander (1961). Also Prasad (1984) reports that ionizing radiation can have different influences on enzyme activity. Among enzymes of which the activity increases after irradiation, most lysosomal enzymes are cited (e.a. acid phosphatase and β-glucuronidase).

The latter mentions radiation doses of 10 Gy, applied on mouse testis. In the present paper we investigated if a correlation exists in our irradiated cartilage between acid phosphatase activity (as a parameter for lysosomal activity) and the inhibition of RNA and PG synthesis.

The most obvious phenomenon in our investigations is the abrupt increase, for each radiation dose, of the number of cells with AP activity, 3 days after irradiation. This is in accordance with the remarks of Prasad (1984), who states that activation of lysosomal activity is not found immediately after irradiation, but that it develops progressively as a function of post-irradiation time. However, the time needed for activation is not mentioned in his paper. The above mentioned phenomenon occurs at the same time the inhibition of RNA and PG synthesis is measured. After 3 days, the continuous increase in positive cells explains the decrease in synthesis during the further culture period.

Dose-dependent differences are less in accordance with the observed radiation response than the time-dependent variations, since we found a higher number of AP positive cells in cartilage irradiated with 50 Gy, compared to cartilage irradiated with 100 Gy. However, looking at the amount of precipitates in each AP positive cell, an irradiation of 100 Gy results at day 7 in an overall distribution of Pb phosphate precipitates, whereas with the dose of 50 Gy, AP activity at that moment is mainly restricted to vacuoles.

In conclusion, we can say that a correlation

exists between the increase in lysosomal acitivity and the observed radiation response of RNA and PG synthesis. Getting an idea about the mechanism responsible for this activation is beyond the reach of this study. The observed decrease in RNA and PG synthesis rather suggests an activation of previously synthesized AP (Hurle and Hinchliffe, 1978). However, the hypothesis that the remaining protein synthesis is of acid hydrolases cannot be ruled out. In that case, an internally programmed mechanism for cell death in our irradiated cartilage must be considered. This kind of cell death, known as apoptosis, is mentioned by several authors in relation to radiation (e.a. Yamada and Ohyama, 1988; Ijiri, 1989; Kerr and Searle, 1980). To find out whether this phenomenon occurs in irradiated cartilage, further investigations are planned.

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Discussion with reviewers

T. Yamada: In the rat thymocytes, it has been said that apoptosis takes place without any change in lysosomal enzymes. However, in tibia cells, activation of AP (acid phosphatase) was found 2 days after irradiation and increased during the whole culture period in this experiment. Please comment on the difference between the thymocytes and tibia?
Authors: Maybe this different behaviour of tibiae is related to their embryonic nature: more specifically we are dealing with embryonic cartilage that, also under normal circumstances, later on differentiates via autolysis and lysosomal participation. In this case irradiation could cause an earlier and accelerated differentiation. To find out if this embryogenesis could be the basis of our observed phenomenon, we are at the moment working on embryonic cartilage that doesn't differentiate into bone, namely embryonic chick sternae. If in this case no relation between irradiation and increase in lysosomal activity is found, our effects are rather related to embryogenesis and differentiation than to cartilage tissue in situ.

S.C. Miller: There is concern that "activation" is not actually being determined as amounts of enzymes were not quantified, only cytochemical distributions. What evidence do the authors have indicating differences in enzyme activation or synthesis rather than differences in intracellular distribution ?
Authors: The method we are using to detect AP is suitable only to demonstrate the "active" enzyme and its distribution. When the enzyme is present in a non-active form, it will not be detected by this method. So if we find alterations in the presence of AP it can be considered as an increase (activation of already present enzyme or new synthesis) in active enzyme.

S.C. Miller: While lysosomes may have some alkaline phosphatase, histochemical studies demonstrate reactivity usually at membranes, particularly the plasma membrane.
Authors: Our method only detects acid phosphatase whose reactivity is demonstrated in most histochemical studies as dense precipitates within lysosomes and vacuoles (autophagic or heterophagic).

R.St.C. Gilmore: Did the authors consider any quantitative method for assessing the amount of AP-positive material present in sections?
Authors: No we only used a cytochemical method. However, by counting the number of AP positive cells and the amount of precipitate in each AP positive cell this method can be seen as a semi-quantitative estimation of the active enzyme.

T. Seed: Were the observations treated statistically?
Authors: No they were not. This approach should be considered as a morphological qualitative and semi-quantitative method to get an idea about the presence of AP.

T. Seed: Are the epiphyseal cells under study a relatively uniform population? Would you characterize them as 'primitive mesenchymal cells'?
Authors: The epiphyseal cells are indead 'relatively' uniform. We would not characterize them as 'primitive mesenchymal cells' because at this stage of the embryo (30 H & H) they synthesize collagen type II and proteoglycan aggregates. So they have to be considered as chondroblasts.

T. Seed: Is there a correspondence (relative to radiation dose/time) between the noted lysosomal disturbances and apoptotic-like nuclear changes?
Authors: What we observe, related to radiation dose and post-irradiation time, is the appearance of indented nuclei. However, to be sure if we are dealing with apoptotic phenomenons, we want to measure cell areas. If in general a shrinkage of the cells is found after irradiation, this speaks for apoptosis. Also the appearance of apoptotic bodies (cell fragmentation) that in our culture system can't be phagocytized, will be checked.

L. de Saint-Georges: The acid phosphatase activity appears here as a logical result of the cell necrosis after irradiation and increasing the number of cell debris that have to phagocytized. Nothing, however proves or even indicates a stimulation of the lysosomal enzymes as a specific consequence of irradiation. The increase of acid phosphatase activity in the non-irradiated tibiae strongly suggests such an interpretation. The additional effects of irradiation in the specimens seems to be due to the increased necrosis which is proportional to time and dose.
Authors: With a radiation dose of 100 Gy and 7 days after irradiation, we see characteristics of necrosis: rupture of cell membranes and consequently an overall distribution of AP. However in the other cases an increase in AP is found before any other morphological alterations could be detected. So the observed time-sequence in the appearance of AP and other morphological alterations speak rather for an apoptosis than a necrotic response.

J.P. Scherft: First, it is stated that the observed stimulation of acid phosphatase activity may explain the way in which irradiation inhibits the synthesis of RNA and proteoglycans. However, it has only been observed that, after irradiation, inhibition of RNA and PG synthesis and stimulation of the activity (or synthesis?) of acid phosphatase run parallelly, and this does not necessarily mean a direct causal relation. I think, there are few arguments in favor of the view that the stimulation of acid phosphatase activity is the primary factor responsible for the decrease of the RNA and PG synthesis. it is much more likely that the increased acid phosphatase activity is a manifestation of an enhanced autophagy, which is a general reaction on cell damage or decreased cell activity. I wonder whether this supposition might be attractive also for the the authors; their remarks as to apoptosis point into that direction.
Authors: Indeed it is more likely to see the enhanced autophagy related to the effects on RNA and PG synthesis. We only want to emphasize the parallelism between AP increase and the observed radiation pattern. It is not possible to define precisely the lysosomal role in our tissue: autolysis may be the cause of cell death (de Duve) but alternatively lysosomal autophagy can be seen as a defence mechanism, as a reaction towards caused damage.
J.P. Scherft: My second point conserns Table I. It is not easy to explain how an irradiation of 100 Gy could result in lower values of acid phosphatase positive cells than irradiation with 50 Gy. One possibility is that higher doses disturb the synthesis of acid posphatase, as they do the synthesis of RNA and PG's, so that the reaction of the lysosomes on the cell damage or at least the diminished activity of the diverse organelles (so the phenomenon of more autophagy) cannot develop. The authors do not discuss this possibility. Can they comment on this?
Authors: We do agree indeed with the point of view that with radiation doses as high as 100 Gy, synthesis of AP is probably disturbed so that reaction of lysosomes to cell damage cannot develop.