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Radioprotective effect of misoprostol on mouse spermatogonial stem cells

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Summary

The radioprotective effects of misoprostol, a synthetic stable analogue of prostaglandin E_1, on spermatogonial stem cells of C3H/HeH × 101/F1 hybrid mice (3H1) were analysed by establishing dose–response relationships for stem cell killing by X-rays in mice that were pretreated with misoprostol. Spermatogonial stem cell killing was studied through determination of the percentage of tubular cross-sections showing repopulation at 10 days after irradiation. In control mice, the D_0 values ranged between 1·7 and 3·6 Gy, dependent on the stage of the cycle of the seminiferous epithelium the cells were in. As found previously, proliferating spermatogonial stem cells were much more radioresistant than quiescent stem cells. In the misoprostol-pretreated animals the spermatogonial stem cells were more radioresistant, the D_0 values ranging from 3·6 to 5·0 Gy. Both proliferating and quiescent spermatogonial stem cells were protected by misoprostol. As the dose–response curves in control and misoprostol-pretreated mice showed about the same extrapolation number to the *y*-axis it was concluded that the misoprostol pretreatment did not alter the kinetics of the repopulation process.

1. Introduction

The experimental evidence in the non-primate testis (reviewed by De Rooij, 1998) favours the model for spermatogonial multiplication and stem cell renewal as originally proposed by Huckins (1971) and Oakberg (1971). In this model there is a compartment of so-called undifferentiated A spermatogonia at the beginning of spermatogenesis. According to their topographical arrangement on the basal membrane of the seminiferous tubules these cells can be subdivided into A_{single} (A_s), A_{paired} (A_{pr}) or $A_{aligned}$ (A_{al}) spermatogonia. The A_s spermatogonia are considered to be the stem cells of spermatogenesis. Upon division of the A_s spermatogonia the daughter cells can either migrate away from each other and become two new stem cells or they can stay together connected by an intercellular bridge and become A_{pr} spermatogonia. The A_{pr} spermatogonia divide further to form chains of 4, 8 or 16 A_{al} spermatogonia. The A_{al} spermatogonia are able to differentiate into A_1 spermatogonia that are the first generation of the so-called differentiating spermatogonia. The A_1 spermatogonia go through a series of six divisions yielding A_2, A_3, A_4, intermediate and B spermatogonia to finally become primary spermatocytes.

Spermatogenesis is a cyclic process. At a certain phase of that cycle the undifferentiated spermatogonia are mostly quiescent and their number is low, only A_s, A_{pr} and a few A_{al} spermatogonia being present. Then the cells are stimulated and for a certain period of time active proliferation occurs, during which increasing numbers A_{al} spermatogonia are formed. The numbers of A_s and A_{pr} spermatogonia do not vary much during the epithelial cycle. The period of active proliferation

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stops as most of the cells become arrested in G_1 phase of the cell cycle. After a subsequent period of relative quiescence almost all the A_{a1} spermatogonia formed during the period of proliferation differentiate into the first generation of differentiating spermatogonia, the A_1 spermatogonia.

It has become clear that the radiosensitivity of spermatogonial stem cells varies greatly during the epithelial cycle. In three different strains of mice, and both X-rays and fission neutron irradiation, it was found that during those stages in which the stem cells are quiescent they are also very sensitive to irradiation, while they are much more resistant in stages in which they are actively proliferating (Van Beek *et al*., 1986; Van der Meer *et al*., 1992, 1993). In C3H/101 F1 hybrid mice the sensitivity for X-rays varies between D_0 values of 1·4 Gy and 2·7 Gy (Van der Meer *et al*., 1993).

Prostaglandins have various protective effects on tissues and cells (reviewed by Needleman *et al*., 1986). Prostaglandins were found to protect the gastric mucosa from ethanol damage. Furthermore, it was demonstrated that various prostaglandins, or synthetic analogues such as misoprostol, protected the mucosa of the digestive tract from injury by non-steroidal anti-inflammatory agents, heat, acids and bases (Robert *et al*., 1979) or X-irradiation (Hanson & Thomas, 1983). Also other cell renewal systems such as bone marrow (Hanson & Ainsworth, 1985; Walden *et al*., 1987) and hair follicles (Geng *et al*., 1992) have been found to become more radioresistant by prostaglandin treatment. The mechanism for the radioprotective effect is unknown. Strong evidence has accumulated that radical scavenging does not play a role (Hanson, 1994) and some data suggest the involvement of DNA repair processes (Zaffaroni *et al*., 1993; Van Buul *et al*., 1997).

We have previously shown enhanced repopulation of the mouse seminiferous epithelium when misoprostol was given shortly before the X-irradiation (Sankaranarayanan *et al*., 1995; Van Buul *et al*., 1997). We now have studied how the misoprostol-induced enhanced repopulation after irradiation is brought about and whether misoprostol protects both proliferating and quiescent spermatogonial stem cells.

2. Materials and methods

(i) *Mice and irradiation*

Adult male C3H/HeH × 101/F1 hybrid mice were obtained from the MRC Radiobiology Unit (Chilton, UK). Irradiation was carried out using an Andrex SMART 225 machine operating at 200 kV and 4 mA and with a measured HVL of 0·2 mm Cu and a dose rate of 0·7 Gy/min. The mice were killed 10 days after irradiation. Control mice were given doses of 1, 2, 3, 4·5 or 6 Gy, 3 or 4 mice for each dose. Misoprostol-treated mice were given 2, 4, 6, 8, 10, 12 or 14 Gy, 4–6 mice for each dose.

(ii) *Misoprostol*

Misoprostol, (±) (16 *RS*)-15-deoxy-16-hydroxy-methyl prostaglandin E_1 methylester, a stable analogue of PGE_1, was kindly made available by Searle (Skokie, IL). It was dissolved in 70% ethanol and stored at −100 °C. Freshly thawed samples were diluted with phosphate-buffered saline to the required concentration prior to the experiment. Thirty minutes before irradiation 20 μg misoprostol was administered intraperitoneally.

(iii) *Histological examination*

The testes were weighed, fixed in Bouin's fluid and embedded in glycol methacrylate (Technovit, Kulzer & Co., Wehrheim, Germany). Five micrometre sections were stained by the periodic acid–Schiff reaction (PAS) and counterstained with Gill's haematoxylin (Polysciences, Warrington, PA).

In each animal, 200 seminiferous tubular cross-sections were examined for the presence of spermatogonia, which was taken as a sign of repopulation. Data were expressed as the percentage of seminiferous tubules showing repopulation: the repopulation index (RI). As shown previously, the RI at 10 days after irradiation correlates well with the number of spermatogonial stem cells surviving the irradiation (Van der Meer *et al*., 1992). Cell counts were performed in epithelial stages I, II–V, VI, VII, VIII–X and XI–XII. Using previously obtained data on the duration of the epithelial stages in 3H1 mice (Van der Meer *et al*., 1993) it was calculated that these stages correspond to stages XI, XII–II, III–IV, IV–VII, VII–IX and IX–X, respectively at the time of irradiation. For example, spermatogonia observed in epithelial stage I derive from surviving stem cells that 10 days earlier, at the time of irradiation, were in an area that was in epithelial stage XI. After doses of 1 and, especially in the misoprostol-treated mice, 2 Gy, the RIs were higher than 80% in cell counts in particular epithelial stages. As this was close to the shoulder of the graphs, RIs of 80% and higher were considered unreliable and were not taken into account in the calculations.

(iv) *Statistical analysis*

Data were analysed using the statistical program SPSS (version 6.1). The presence of a significant linear term and/or a significant quadratic term was tested in a single classification analysis of variance, in which it was assumed that data points were independent and normally distributed. For the calculation of the D_0 of the stem cells, data were fitted to the model $\ln(A\text{-})$

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$= - \propto D$ by means of a weighted linear least-squares regression method. The reciprocals of $\ln(A)$ variances were used as weighting factors. A represents the repopulation index on day 10 after irradiation, found after a radiation dose D, and $\propto$ represents $1/D_0$.

Data were also examined for the presence of a quadratic term by fitting them to the linear-quadratic model $\ln(A) = -(\propto D + \beta D^2)$, which can give an indication of a shoulder in the dose–response curve. When both the linear and the quadratic term were significant, the $\propto$ and β coefficients were calculated.

3. Results

Ten days after irradiation at doses of 4 and 6 Gy, testis weight of the misoprostol-pretreated mice was slightly but significantly higher than in the irradiated control mice (Fig. 1).

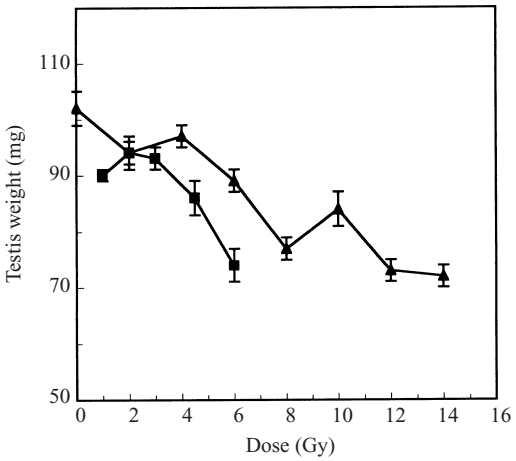

Fig. 1. Testis weight (mean ± SEM) of mice 10 days after receiving graded doses of X-rays. Triangles, misoprostol-pretreated mice; squares, control mice.

To study the effects of misoprostol treatment in more detail the RIs were determined in the testes of irradiated control and misoprostol-pretreated mice. With increasing dose the RIs decreased faster in areas that at the time of irradiation were in epithelial stages VI–X than in other stages in both the controls and the misoprostol-treated mice (Fig. 2, Table 1), indicating more radiosensitive populations of spermatogonial stem cells in these stages. Furthermore, comparing similar stages the RIs were always clearly higher in the misoprostol-treated than in the control animals.

A weighted linear regression analysis was performed for each stage. The reciprocals of the variances of the log-transformed cell count data were used as weighting factors. In all cases there was a significant linear term. In this way, radiosensitivities could be estimated for stem cells in the various epithelial stages (Fig. 3, Table 1). The extrapolation numbers of the dose–response graphs to the y-axis were similar for control and misoprostol-treated mice (Table 1).

4. Discussion

The dose–response relationships found for the killing of spermatogonial stem cells in control 3H1 mice confirm those described previously (Fig. 3; Van der Meer *et al.*, 1993). All D_0 values presently calculated for the various epithelial stages in the control group fall within the 95% confidence ranges observed in the previous, more detailed study. Again the quiescent spermatogonial stem cells present in epithelial stages VI–X were found to be much more radiosensitive than the proliferating stem cells present in the other stages.

As already suggested by a smaller loss of testis weight, misoprostol pretreatment had a radio-protective effect on spermatogenesis. Pretreatment of the mice with misoprostol clearly made the

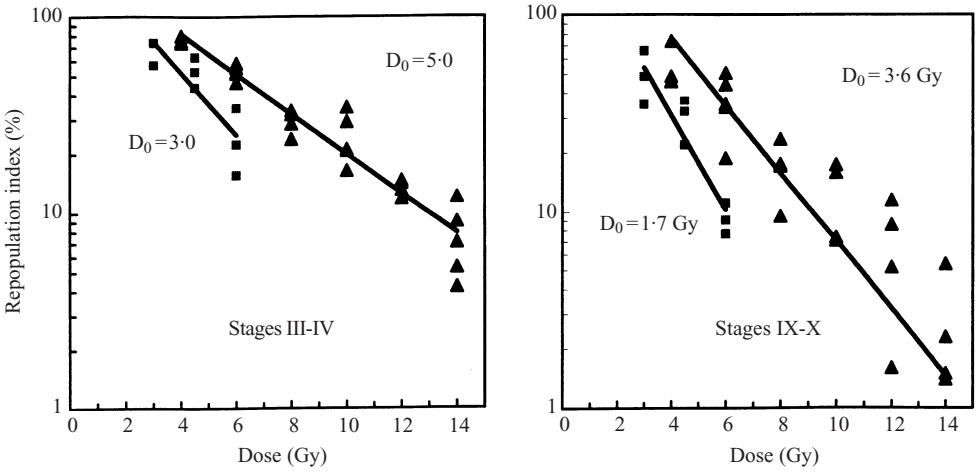

Fig. 2. The effects of misoprostol on the dose–response relationships of spermatogonial stem cells in stages with a high (stages IX–X) and a low radiosensitivity (stages III–IV) of the seminiferous epithelium, for the cell killing effects of X-irradiation. The stages indicated are the stages at the time of irradiation. Triangles, misoprostol-pretreated mice; squares, control mice.

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Table 1. *Radiosensitivity of spermatogonial stem cells in various stages of the cycle of the seminiferous epithelium as expressed by their D_0 value, with 95% confidence limits (range), for cell killing*

Epithelial stages at irradiation	Control			Misoprostol-pretreated		
	D_0 (Gy)	R^2	Extrapolation no.	D_0 (Gy)	R^2	Extrapolation no.
XII–II	3·6 (2·8–4·9)	0·65	165 (122–224)	4·6 (4·5–4·7)	0·98	184 (177–191)
III–IV	3·0 (2·8–3·3)	0·95	177 (156–200)	5·0 (4·6–5·6)	0·79	140 (123–159)
IV–VI	2·8 (2·5–3·2)	0·86	131 (117–146)	4·3 (4·0–4·7)	0·82	102 (95–109)
VI–IX	1·8 (1·7–1·9)	0·98	156 (139–174)	3·6 (3·4–3·9)	0·91	115 (106–124)
IX–X	1·7 (1·5–2·0)	0·89	333 (223–497)	3·6 (3·2–4·1)	0·77	165 (135–203)
XI	2·3 (2·0–2·5)	0·92	249 (201–309)	3·8 (3·7–4·0)	0·97	215 (204–226)

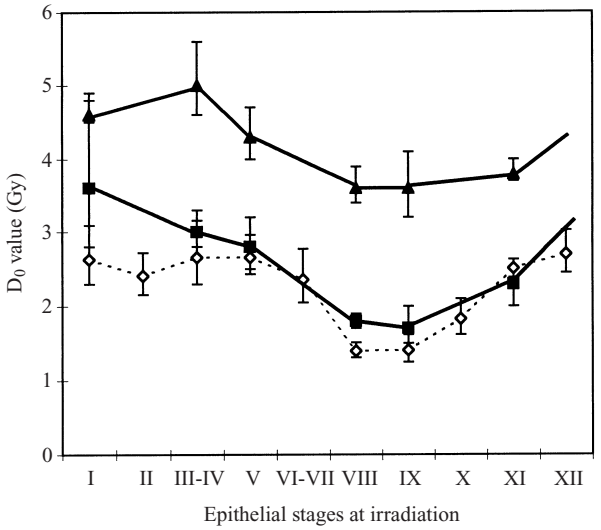

Fig. 3. The D_0 values for the induction of spermatogonial stem cell death by acute X-rays in 3H1 mice and their 95% confidence intervals, in various stages of the cycle of the seminiferous epithelium. Triangles, misoprostol-pretreated mice; squares, control mice; dotted line, data from Van der Meer *et al.* (1993) for control 3H1 mice.

spermatogonial stem cells more radioresistant. This beneficial effect of misoprostol was observed in all stages of the epithelial cycle. Apparently, the protective effect of misoprostol is independent of the proliferative activity of the cells as both quiescent and proliferating spermatogonial stem cells became more radioresistant towards cell killing by X-irradiation. The average increase in the D_0 values when misoprostol pretreatment was given (a factor of 1·7) is comparable to the 1·5-fold increase in survival of Syrian hamster embryo cells found by Miller *et al.* (1994).

While misoprostol clearly increases the radioresistancy of the stem cells, a higher RI at a particular interval after irradiation can theoretically also be brought about by increased proliferative activity of the surviving stem cells after irradiation. Increased proliferation of the surviving stem cells will result in larger repopulating colonies occupying greater lengths of seminiferous tubules and consequently a higher RI. This would cause a shift upwards of the dose–response graphs and upon extrapolation these graphs would cross the *y*-axis at a higher level. However, the extrapolation numbers calculated were rather similar (Table 1) and did not give any indication that this had happened. Apparently, the misoprostol pretreatment did not affect the proliferative activity of the surviving stem cells.

The present results raise the question whether or not it would be possible to protect the seminiferous epithelium of patients from radiation damage by giving an intratesticular injection of misoprostol prior to irradiation. To address this question a preliminary experiment was done in which rats received an intratesticular injection of 1 or 2 μg of misoprostol before receiving a dose of 7 Gy of X-rays to the testes. No significant protection was observed, which could either be caused by the dose being too low or the possibility that upon intratesticular injection misoprostol does not spread well throughout the testis (De Rooij *et al.*, unpublished). This needs to be studied in further detail.

In conclusion, the enhanced repopulation of the seminiferous epithelium after irradiation following pretreatment with misoprostol, as shown previously (Sankaranarayanan *et al.*, 1995; Van Buul *et al.*, 1997), is caused by a decrease in the sensitivity of spermatogonial stem cells towards the cell killing effects of X-irradiation in all stages of the cycle of the seminiferous epithelium. In addition, previous results indicated that spermatogonial stem cells also become less sensitive towards the induction of reciprocal translocations by higher doses of X-irradiation (Sankaranarayanan *et al.*, 1995). So far only prostaglandin-mediated protection responses of *in vivo* cell renewal systems as a whole, such as bone marrow (Hanson & Ainsworth, 1985; Walden *et al.*, 1987), hair follicles (Geng *et al.*, 1992), intestine (Hanson & Thomas, 1983) or germinal epithelium, have been described. In the present study we have further dissected this response and show for the first time that the protective effect is clearly present in the stem cell compartment – and, moreover, for both

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proliferating and quiescent stem cells. This observation supports the use of misoprostol for protecting normal tissues in cancer therapy, the first clinical trials of which have been started (Hanson *et al*., 1995).

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