Effects of gamma irradiation on dermal equivalents in vitro

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Summary. Dermal equivalents (DE), collagen lattices, were produced in vitro and used as a model for studying the possible role of a pure population of fibroblasts in post-radiotherapeutic dermal fibrosis. Single doses of gamma irradiation induced a partial inhibition of the collagen lattice retraction and of protein synthesis. The collagen production was less inhibited than was synthesis of non-collagen protein, which resulted in an increase of the relative amount of collagen synthesized by irradiated fibroblasts. These data suggest that gamma irradiation might be able to select some fibroblast clones able to produce increasing amounts of collagen. This selection process could be involved in the development of tissue fibrosis after therapeutic radiation.
Key words. Dermal equivalent model; collagen lattices; three-dimensional fibroblast cultures; gamma irradiation; collagen and protein synthesis.

The in vitro culture of human fibroblasts in three-dimensional collagen gels (or lattices) was first described by Bell et al. in 1979[1]. In this model, cells progressively reduce the size of the collagen network. This behavior is considered to be a general attribute of normal fibroblasts[2]. As in vivo, the slow proliferation rate and the reduced collagen synthesis are characteristic features of these 'dermal equivalent' (DE) cultures[3]. Post-radiotherapeutic fibrosis of the skin is a major complication of gamma irradiation[4]. It is likely that fibroblasts play a role in this phenomenon, since these cells are responsible for the collagen synthesis in the dermis and because the accumulation of collagen in fibrotic tissue has been demonstrated[5]. Activation of fibroblasts may be indirect, through the release of a series of cytokines by infiltrated inflammatory cells[6], but the possibility of a direct effect of gamma rays on fibroblasts activation has not yet been studied, especially in 3-dimensional structures such as DE cultures, which are highly homologous with the in vivo situation. In this paper, the DE model was used to provide further data on the retraction of the collagen gel, the kinetics of cell growth and the protein synthesis capacities of human skin fibroblasts after gamma irradiation at various dose levels.

Materials and methods

Tissue culture media were obtained from Gibco, except fetal bovine serum (FBS) which was from IBF. Plastic culture flasks were from Nunc. Collagen was prepared in the laboratory by pepsin digestion of calf skin according to Fujii and Kühn[7]. The collagen was sterilized by washing with 70% ethanol and solubilized at a concentration of 2 mg/ml in sterile acetic acid 0.018 M. *Clostridium histolyticum* collagenase (CLSPA grade) was obtained from Worthington and purified in the laboratory, β-aminopropionitrile (fumarate) was from Sigma, ascorbic acid from Merck and proline from Calbiochem. ^{14}C proline (specific activity: 9.25 GBq/mmol) was provided by CEA. Other reagents, of the highest purity available, were from Prolabo. For lattice irradiation, a cobalt 60 source from CEA with an output of 2.3–2.6 Gy per min was used. Quality control dosimetry was carried out using both an ionizing chamber (Ionex from Nuclear Enterprise) and thermoluminescent dosimeters.

Fibroblast cultures were established from explants of foreskins from healthy young children. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (400 UI/ml), streptomycin (50 µg/ml) and 10% fetal bovine serum, and grown in plastic culture flasks in a 5% CO_2, 95% air atmosphere at 37 °C. Confluent monolayers were propagated by trypsinization and replated at 1:4 dilution. Cells used in the experiments reported here had been passaged 3–8 times in vitro. The lattices were prepared in bacteriological petri dishes according to the procedure described by Gillery et al.[8]: 1.8 ml of 2.5 x concentrated medium was combined with 0.5 ml of FBS, 0.7 ml of 0.041 M NaOH, 1.5 ml of collagen solution (2 mg/ml) and 0.5 ml of cell suspension (400,000 cells/ml). At 37 °C, the mixture polymerizes in less than 10 min. 15 min after gel formation, irradiation of the dishes was carried out and the dose delivered to the lattice at the 100% isodose level varied from 5 Gy to 30 Gy in one fraction. The diameters of the lattices were measured every day following irradiation for 7 days, using a linear calibrated scale. To determine the number of fibroblasts, the lattice was digested using clostridial collagenase (7.5 U per lattice for 90 min at 37 °C). After incubation, the cells were freed and counted using a Neubauer hemacytometer. The viability of the fibroblasts was estimated by the trypan blue test.

For protein synthesis measurements, the DE cultures were incubated for 24 h in DMEM supplemented with 10% FBS, 0.2 mM β-aminopropionitrile, 0.28 mM ascorbic acid, 2 mM glutamine and 74 KBq/ml ^{14}C-proline diluted into 200 µM cold proline. At the end of the

incubation period, the lattices and the culture media were collected separately. After several washes in PBS, the lattices were dissolved in 0.2% Triton X100. The culture media and solubilized (lattice + cells) fraction were then dialyzed extensively to eliminate ^{14}C proline not incorporated into proteins. ^{14}C proline incorporation into total proteins and collagen was then measured in both fractions using the collagenase digestion method described by Peterkofsky and Diegelmann[9] adapted by Gillery et al.[10]. The percentage of collagen to total proteins was measured according to the following formula[11]: % collagen = (cpm CDP) × 100/[cpm CDP + (5.4 cpm NCP)] where CDP means 'collagenase digestible proteins' and NCP 'non-collagen proteins'. The experiments were done in quadruplicate and expressed as mean plus or minus one standard deviation. The statistical analysis was done using Student's t-test.

Results

Gamma irradiation induced a slight but significant dose-dependent decrease of the intensity of the collagen gel retraction, the gel shrinkage being inversely proportionate to the size of the dose (table 1). In DE irradiated at 5 Gy, the diameter of the gel was significantly larger on day 6 than that of the non-irradiated DE ($p < 0.05$). In DE irradiated at 10 Gy, 20 Gy and 30 Gy, a significant difference was observed earlier, on day 5. After 7 days, in non-irradiated control gels the lattices had progressively contracted to 81% of their original area. In irradiated gels, the final area was 78%, 77%, 76% and 75% of the original for 5 Gy, 10 Gy, 20 Gy and 30 Gy irradiation respectively. The mean diameters were 9.4 +/− 0.5 mm, 10.9 +/− 0.25, 11.33 +/− 0.3, 12 +/− 0.25, and 12.6 +/− 0.5 mm.

For the study of the kinetics of cell growth (fig. 1), the lattices were only irradiated at 0 Gy (controls), 10 Gy and 30 Gy on day 0. The cell numbers were measured every day from day 1 to day 7. In control DE, initial seeding was followed by a decrease of the cell number per lattice which lasted for the first 3 days. After the third day, cell proliferation occurred and the final number of fibroblasts in the DE was then significantly higher than the initial number of seeded cells (249,500 +/− 24,000 vs 200,000 +/− 10,000). In irradiated DE, the inital decrease on day 1 was also observed. The most striking difference was that irradiated fibroblasts did not proliferate, their number remaining statistically constant until day 7, and lower than the number of cells initially seeded: 175,000 +/− 14,000 and 149,000 +/− 9300 on day 7 in 10 Gy and 30 Gy irradiated DE. The percentage of non-viable cells measured by the trypan blue test was 0% for controls, 5.5% at 10 Gy and 7.3% at 30 Gy.

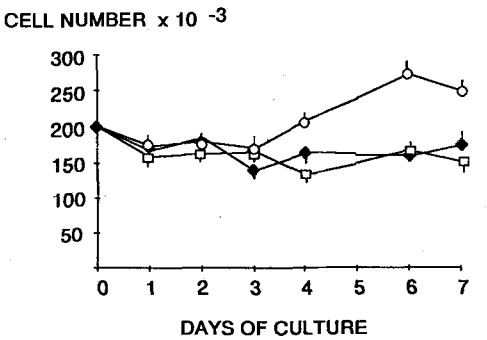

Figure 1. Cell number measurements in lattice cultures not irradiated (○), irradiated with 10 Gy (◆) or with 30 Gy (□). Results are means of quadruplicate experiments +/− 1 standard deviation. 200,000 fibroblasts were seeded in every lattice on day 0.

The biosynthetic activity of the fibroblasts was measured on day 7 when contraction was complete (fig. 2). Total protein synthesis estimated as ^{14}C-proline incorporation decreased in all irradiated cultures. The decrease was more severe in the lattice than in the culture medium: the synthesis of total proteins was reduced to 50% and 33% at 5 Gy, and to 70% and 45% at 30 Gy, respectively. In both gel and culture medium, the difference was statistically significant at 2.5 Gy ($p < 0.05$). In contrast, the collagen synthesis was less altered, and the decrease became significant from 10 Gy, but not for smaller doses. As a consequence, the relative amount of collagen compared to total proteins increased (table 2).

Table 1. Effect of gamma irradiation on the contraction of collagen lattices due to human fibroblasts. Each value is the mean of quadruplicate determinations of the surface of the lattice (in mm^2) +/− 1 SEM. 200,000 cells were seeded per lattice. Irradiation was carried out on day 0, when all the lattice surfaces were identical and equal to the surface of the dish (diameter = 50 mm; surface: 1963.5 mm^2).

		Control	5 Gy	10 Gy	20 Gy	30 Gy
D1	Mean	628	587	534	642	575
	+/− SEM	56	112	98	116	89
D2	Mean	146	149	150	162	163
	+/− SEM	10	14	6	11	14
D3	Mean	123	118	120	128	133
	+/− SEM	11	10	22	10	17
D4	Mean	102	106	113*	123*	130*
	+/− SEM	9	4	0	8	6
D5	Mean	80	102*	113**	123**	125**
	+/− SEM	4	4	0	11	10
D6	Mean	69	93*	101**	118**	125**
	+/− SEM	2	7	4	5	10

*$p < 0.05$; **$p < 0.01$.

Table 2. Collagen synthesis as a percentage of total protein synthesis in control and irradiated cultures after ^{14}C-proline incorporation. 200,000 cells were seeded per lattice on day 0 and irradiated 15 min after lattice formation. Data are deduced from the results presented in fig. 2.

Dose delivered	Percentage of collagen synthesis
Experiment 1	
0 Gy (control)	1.36
1 Gy	1.47
2.5 Gy	1.80
5 Gy	2.69
Experiment 2	
0 Gy (control)	1.84
10 Gy	2.27
20 Gy	2.39
30 Gy	2.31

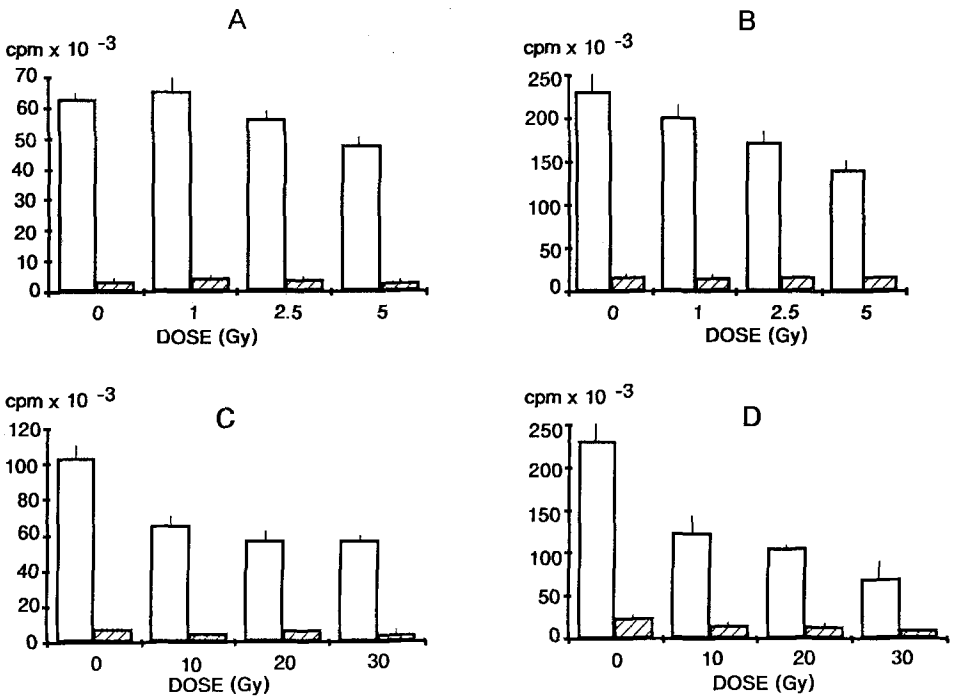

Figure 2. Measurement of ^{14}C-proline incorporation into total proteins (open bars), collagen (hatched bars) synthesized by lattice cultures (2 different experiments). Results are mean of quadruplicate experiments +/− 1 standard deviation. *A* and *C*: culture medium; *B* and *D*: lattice + cells; *A* and *B*: irradiation from 0 to 5 Gy (experiment 1); *C* and *D*: irradiation from 0 to 30 Gy (experiment 2).

Discussion

Cultures of fibroblasts in collagen lattices constitute a valuable tool for the study of the behavior of cells embedded in an extracellular matrix. In these culture conditions, comparable to the physiological in vivo situation, cells divide slowly and synthesize less collagen than in monolayers. The most striking feature of the lattice cultures is the contraction which occurs during the first days and results in the structuring of a resistant, organized and coherent network considered as a dermal equivalent[12]. Therefore, DE could be a better model than monolayer cultures for studying the effects of gamma irradiation of dermal cells and its relationship with dermal fibrosis. Such a DE model has never been used for that specific purpose.

In the DE model, it is generally accepted that the retraction of the collagen network is a function of cell number initially seeded[2,10,13]. The slight but significant dose-dependent inhibition of retraction observed after irradiation may be explained by radio-induced cell reduction, correlated to cell killing. Results of experiments on cell proliferation in collagen gels have been contradictory: no significant proliferation[10,14], or a significant proliferation occurring during the first days of culture[15], or a slight proliferation starting on day 4 (present results). For that reason, the importance of radio-induced mitotic cell killing is difficult to estimate. Nevertheless, we found no proliferation of the cells after irradiation. The difference in the final cell number may be responsible for the late inhibition of retraction capacity since it appeared only on day 4, whereas most of the retraction occurred during the first days. The exact influence of cell number on lattice retraction is still being discussed. Allen and Schor[16] demonstrated that in non-irradiated gels, the final diameters after 10 days of incubation were independant of initial cell density provided that more than $9x10^4$ cells were seeded. Nishiyama et al.[13] observed that when 100,000 and 200,000 fibroblasts were seeded, the rate and the intensity of retraction did not differ significantly. However, these groups did not use the same culture conditions as ours. In our model[8,10], the lattice retraction was always closely related to the number of seeded cells. The same results were obtained by other authors[1,17,18]. Nevertheless, it cannot be excluded that gamma-irradiation might decrease the retraction ability of cells through an effect on their cytoskeleton.

The occurrence of radio-induced fibrosis may be due either to an increase in the number of cells capable of synthesizing collagen, or to an increase in the amount of collagen synthesized per cell[4]. As expected, the reduction in the cell number after gamma irradiation was correlated with a reduction in total protein synthesis. Our results, showing a relative increase of collagen synthesis compared to other protein production, suggest that gamma irradiation might induce the selection of some fibroblast clones able to produce more collagen than in normal conditions. Another possibility is that collagen synthesis in a given cell is less sensitive to irradiation than the synthesis of other proteins. In monolayer cultures, the existence of various fibroblast populations which differ

in their potential for collagen synthesis has been demonstrated by several authors[19-21]. Our hypothesis is in agreement with previous histological observations[22] and the findings published by Wegrowsky et al.[23] showing an increase in collagen content and synthesis in fibroblast cultures established from samples of irradiated fibrotic tissue. Another hypothesis would be the release from irradiated DE of some activator(s) of collagen synthesis.

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Phenytoin-induced DNA synthesis and inositol 1,4,5-trisphosphate formation in L-929 fibroblasts

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Summary. Culture of L-929 fibroblasts in the presence of phenytoin (2.5–5.0 μg/ml) increased DNA synthesis, as indicated by increased [^{3}H]thymidine uptake, while a higher dose (20 μg/ml) inhibited DNA synthesis. In like manner, a low dose of phenytoin (5.0 μg/ml) was effective in increasing inositol 1,4,5-trisphosphate formation while a higher dose (10 μg/ml) tended to inhibit this activity. These data suggest that the formation of inositol phosphate second messengers may play a role in phenytoin-induced fibroblast proliferation and connective tissue growth.
Key words. Phenytoin; fibroblasts; inositol phosphates; DNA synthesis.

Phenytoin (5,5-diphenylhydantoin) has been used as an effective drug for the control of seizures since its clinical introduction in 1938[1]. Since that time phenytoin therapy has been associated with overgrowth of gingiva in about half of those receiving the drug[2] and less frequently, excessive connective tissue growth in other areas[3-5]. Further, phenytoin has been shown to be mitogenic for fibroblasts in vitro[6-8]. However, the mechanism by which phenytoin induces connective tissue growth or mitosis of fibroblasts has not been determined. The relationship of some mitogens to the receptor-coupled hydrolysis of membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) to form the intracellular 'second messengers', inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol[9-11] led us to compare the mitogenic properties of phenytoin and its possible activation of the phosphoinositide transmembrane signal mechanism in cultured fibroblasts. We report here that a critical in vitro concentration of phenytoin (within the range of therapeutic blood levels) increased both the rate of DNA synthesis and cellular levels of IP_3 and its metabolites, inositol 1,4-bisphosphate (IP_2) and inositol 1-phosphate (IP_1), in L-929 fibroblasts.

Materials and methods

Cell culture and assessment of mitogenic properties of phenytoin. The mitogenic activity of phenytoin in vitro was assessed by determination of [^{3}H]thymidine incor-