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THE EFFECT OF IONIZING IRRADIATION ON TYPE I COLLAGEN OF THE TAIL IN GROWING MICE: A HISTOLOGY AND ELECTRON MICROSCOPY STUDY

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Abstract

In order to examine the effect of radiation on growing tissue, especially the fibroblasts and their end-product, the collagen fibres, tails from 24 mice were irradiated at an age of 8 days with 20 Gy and 30 Gy (^{60}Co). Tails from 18 animals served as controls. Six mice from each group were sacrificed on day 8, 20 and 30. Transmission electron microscopy was used to examine the fibroblasts and the collagen fibrils. Non-irradiated fibroblasts had a nucleus rich in chromatin and an abundant endoplasmic reticulum with cisternae and condensing vacuoles.

On day 20, approximately 50%, and on day 30, 25% of the fibroblasts irradiated with 30 Gy had a sparse endoplasmic reticulum pointing to a reduction of protein synthesis. While, on day 20, the fibrils irradiated with 20 Gy and with 30 Gy had significantly larger diameters compared to the controls, on day 30, the irradiated fibrils had a notably smaller diameter compared to the controls; 30 Gy-fibrils were larger than the 20 Gy-fibrils on both days.

On day 20, the binding mean value of the 30 Gy-fibrils exceeded that of the controls and was significantly higher than that of the 20 Gy-fibrils, which was lower, though not significantly, than the controls. On day 30, the banding mean value of the 30 Gy-fibrils was notably lower than the control; and the value of the 20 Gy-fibrils was significantly lower than that of the 30 Gy-fibrils. The results are explained as an edema together with an inhibitory effect on the protein synthesis of the fibroblasts caused by the irradiation. This deduction is further supported by light microscopy of the tails.

Key Words: Collagen fibrils, ionizing irradiation, mice, transmission electron microscopy.

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Introduction

For several decades, radiobiologists have sought to elucidate changes in cell kinetics, both in neoplastic and normal tissue. However, both in published reports and in textbooks, slow-growth structures, and structures lacking replicative capacity {e.g., connective tissue (collagen), an end product of fibroblast activity}, have been considered of secondary importance with regard to the elucidation of the physiological effects of ionizing radiation on living tissue. It is true that collagen has been the object of limited radiobiological interest, even in such radiotherapeutic contexts as pulmonary fibrosis. Collagen metabolism in lung tissue has been investigated in animal studies where fibrosis was artificially induced, e.g., by inhalation of radioactive gas in a dog model (Pickrell *et al.*, 1975) or in mouse models (Talbot and Moores, 1985; McAnulty *et al.*, 1991), or by exposure of the lungs to X-rays in a mouse model (Murray and Perkins, 1987). In all these studies, exposure to radiation was shown to increase collagen synthesis but with concomitant signs of degradation. Another context where the effect of radiation on collagen has been investigated is sterilization with ^{60}Co (De Deyne and Haut, 1991), though in such studies the dosages were in the Mrad range (1 Mrad = 10^4 J/kg), a level of little interest in radiotherapy.

The aim of the present study was to examine the *in vivo* response of collagen Type I (in the mouse tail tendon) to ionizing radiation. To this end, both the effect of radiation on fibroblasts and the diameter of the fibrils were investigated at different ages and irradiation doses; the fibril band length also was calculated. This approach constitutes an expansion of previous experiments by Jonsson *et al.* (1985), who showed growth to be retarded in mouse tails exposed to ionizing radiation. However, in contrast to the study by Jonsson *et al.* (1985), in the original findings, all the tissues in the irradiated tails appeared to have been affected. The present study utilizes histology together with transmission electron microscopy (TEM) to interpret the findings.

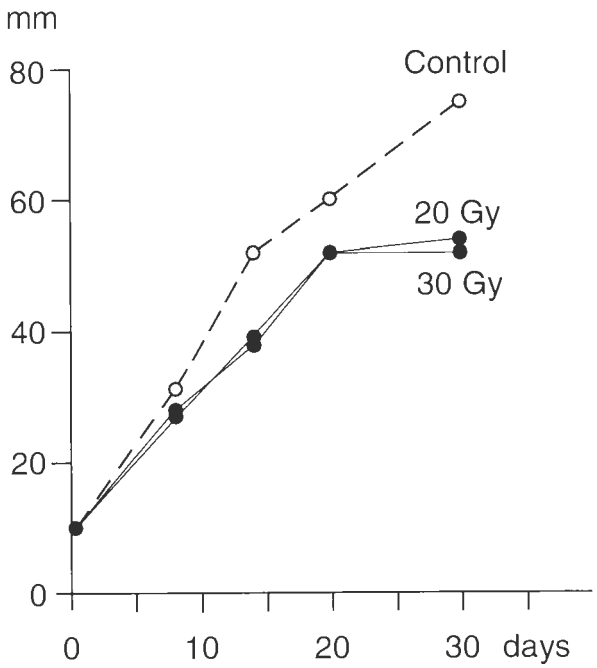


Figure 1. Tail length in millimeters of the control and irradiated animals at different intervals after birth.

Material and Methods

Animals

Fifty-four BALB/c mice, bred at the laboratory, were used for the experiments. At 8 days of age, 6 mice were sacrificed for examination before any treatment began. Of the remaining 36 animals, 12 were irradiated with 20 Gy, 12 with 30 Gy on day 8, and 12 non-irradiated mice served as controls. Six animals from each group were sacrificed on day 20, and the remaining 18 mice on day 30. In the following, the controls are referred to as the 8-day, 20-day and 30-day control groups, the irradiated animals comprising 20-Gy/20d and 20-Gy/30d, 30-Gy/20d and 30-Gy/30d groups.

Preparation for transmission electron microscopy

After sacrifice, the mouse tail was cut off and fixed by immersion for 24 hours in 2.5% glutaraldehyde in cacodylate buffer (pH 7.2). After fixation overnight, the specimens, all taken from the middle part of the tail, were postfixed in osmium tetroxide in the same buffer, dehydrated, contrasted en bloc in phosphotungstic acid and uranyl acetate and embedded in epoxy plastic. Semi-thin sections (1 μm thickness) were stained with methylene blue and used for light microscopy. Ultrathin sections were contrasted with lead citrate and uranyl acetate for examination by TEM (JEOL CX 100, JEOL USA, Peabody, MA). Paraffin sections (6 mm thick) were made from the tail cut longitudinally in the midline and transversely, proximal to the TEM specimen.

Tail length

Tail length was measured and expressed in millimeters. Measurements were made at birth and on days 8, 14, 20 and 30 after birth.

Examination of fibroblasts

Using TEM at magnifications varying from 3,000x to 50,000x, fibroblasts were examined in cross-sectional preparations from every animal; 5-8 micrographs were taken from 5 of the 6 animals in each group. This examination, which was not part of the original protocol, was included due to the manifest difference between irradiated and non-irradiated fibroblasts. As a result, the fibroblasts were divided into two protein synthesis subgroups: one designated high-activity, the other low activity on the basis of morphological findings. Thus, 20 fibroblasts from each tail were evaluated, and 5 animals from each radiation and control group, giving a total 100 fibroblasts per group, which was considered to provide a satisfactory statistical basis for assessing any difference between the irradiated and non-irradiated groups.

Calculation of fibril diameter

The cross-sectional diameter of collagen fibrils was measured with a digital slide caliper on four micrographs from each of 5 animals of the 6 animals in each group (i.e., a total of 200 fibrils). Thus, each group of five animals is represented by 1000 measurements. In each micrograph, the magnification was 60,800x (instrumental magnification was 38,500x).

Calculation of fibril band length

From each of the collagen fibrils examined, 50 periods (band lengths) were measured with a slide caliper (with 0.01 calibrations) on micrographs at magnifications of up to 80,000x (instrumental magnification was 38,500x or 42,000x). Ten fibrils were examined in each animal, making a total of 3000 periods for each group of six mice; values were expressed in nanometers (nm).

Radiation

^{60}Co irradiation of mouse tail was delivered with a Siemens (Darmstadt, Germany) Gammatron S at a distance of 80 cm and a dose rate of 1.0 Gy/min. Unanesthetized mice were positioned in a plexiglass jig capable of handling five mice at once; the bodies of the mice were shielded with lead. The mouse tails were extended in the radiation field and covered with a 5 mm plexiglass sheet to ensure full build-up. Dosimetry was performed with a thermal luminescence dosimeter (TLD).

Results

Tail length

Figure 1 shows the tail length curves of the controls and both irradiated groups. In the series as a whole

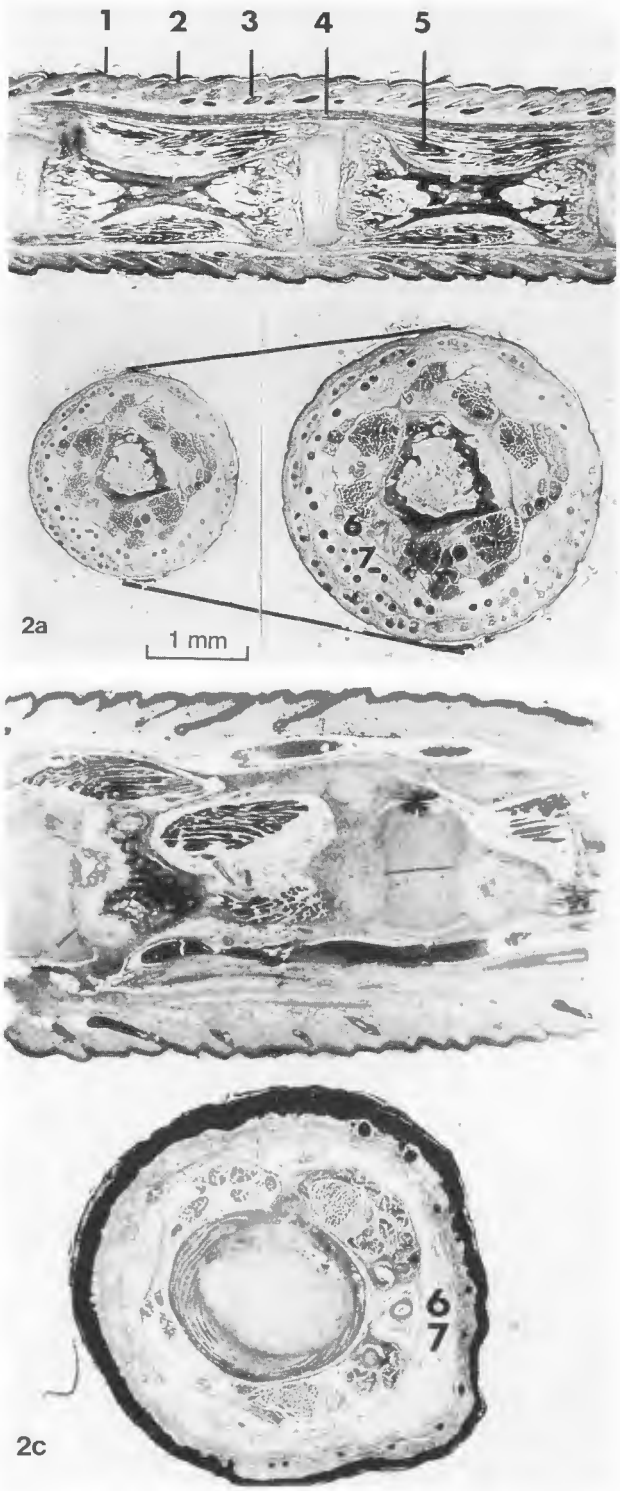


Figure 2. Paraffin sections, longitudinal and crosswise, of the mouse tail at 30 days, at identical magnification. **(a)** Non-irradiated control, with the transverse section enlarged; irradiated, 20 Gy **(b)** and 30 Gy **(c)**. **1**: stratum corneum; **2**: stratum lucidum; **3**: hair follicle; **4**: muscle fibers; **5**: collagen bundles; **6**: artery; **7**: vein.

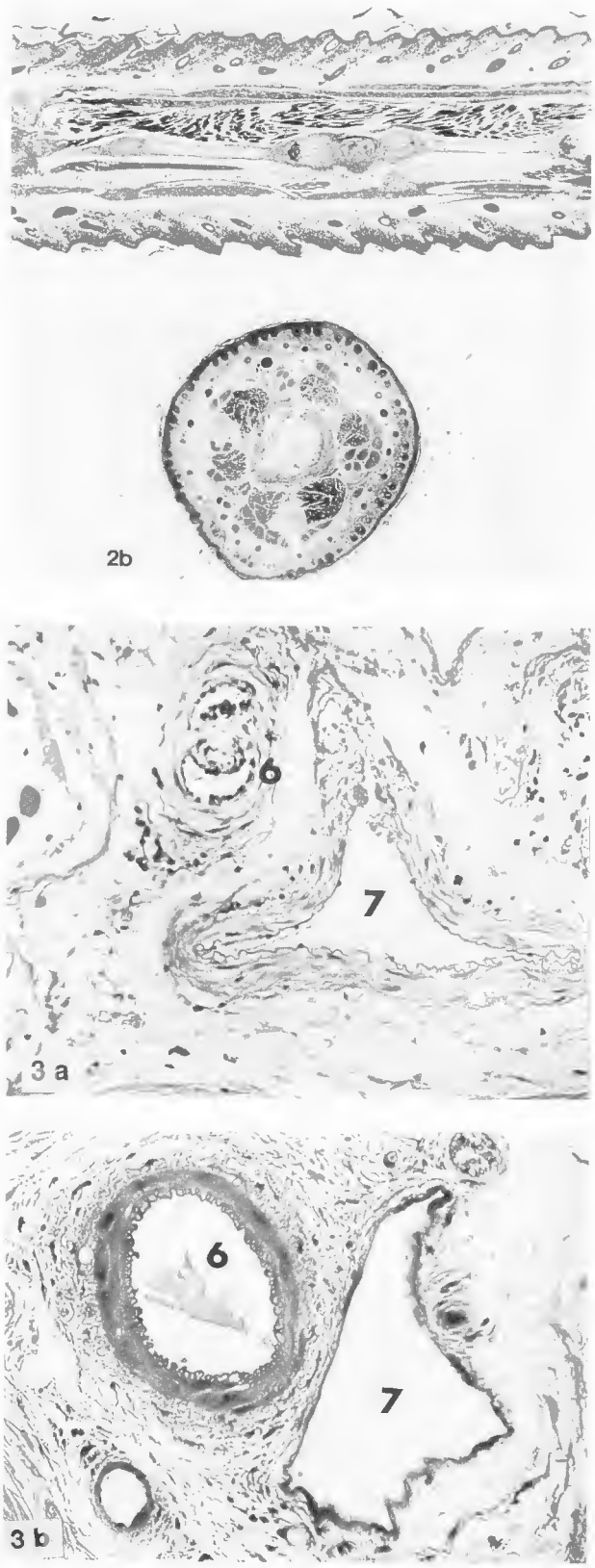
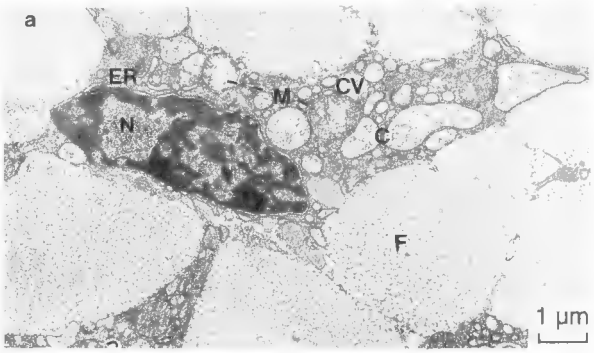
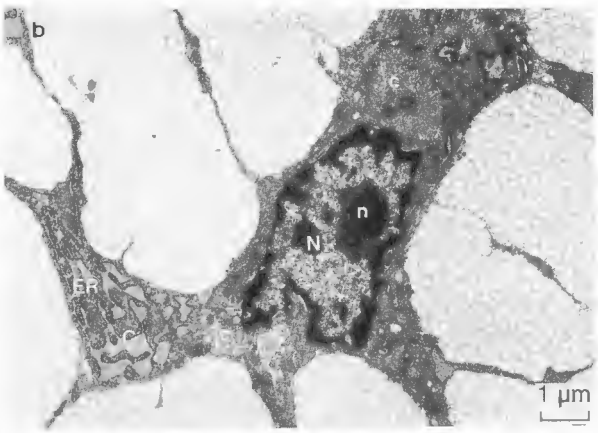
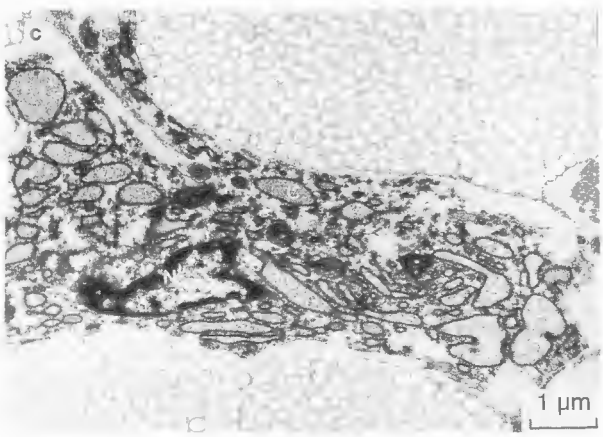


Figure 3. Twenty-fold enlargements of artery (6) and vein (7) from: **(a)** Figure 2a; and **(b)** Figure 2c.







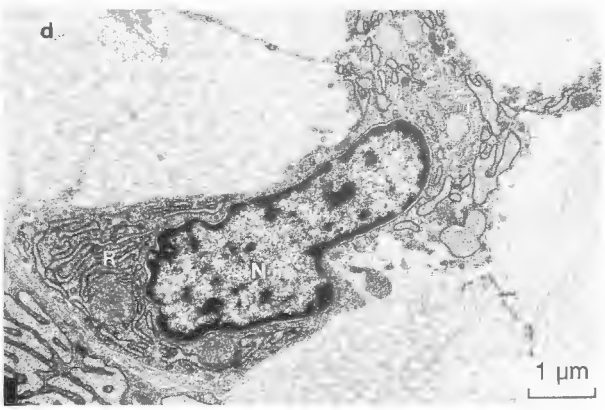
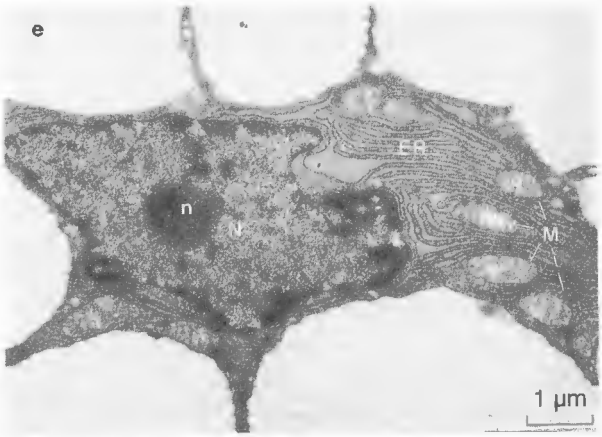
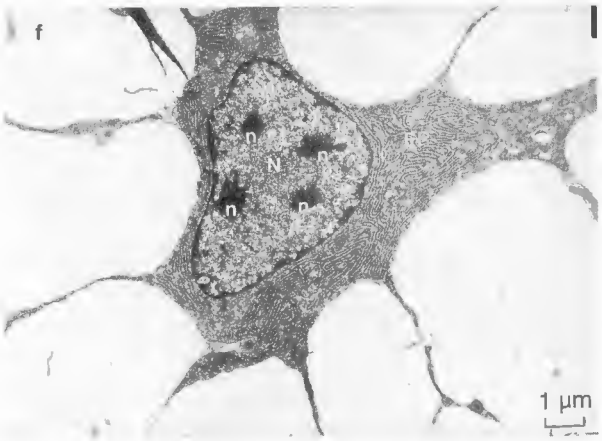
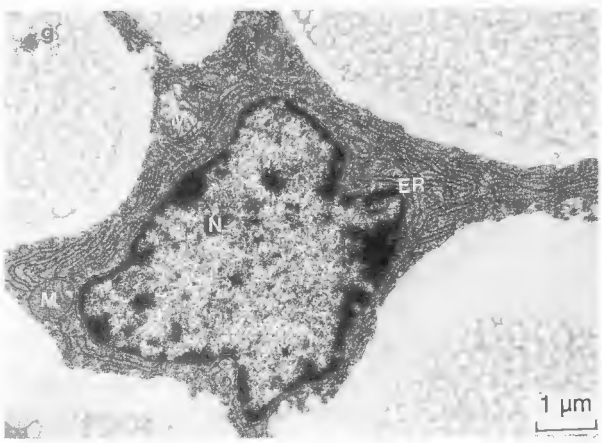


Figure 4. Fibroblast cells from the different groups. N: Nucleus with chromatin; n: Nucleolus; ER: Endoplasmic reticulum; C: Cisternae; R: Ribosomes; CV: C vacuole; F: Collagen fibrils; M: mitochondria. **(a)** Control 8 days. **(b)** Control 20 days. **(c)** Control 30 days. **(d)** 20-Gy/20 days. **(e)** 20-Gy/30 days. **(f)** 30-Gy/20 days. **(g)** 30-Gy/30 days.







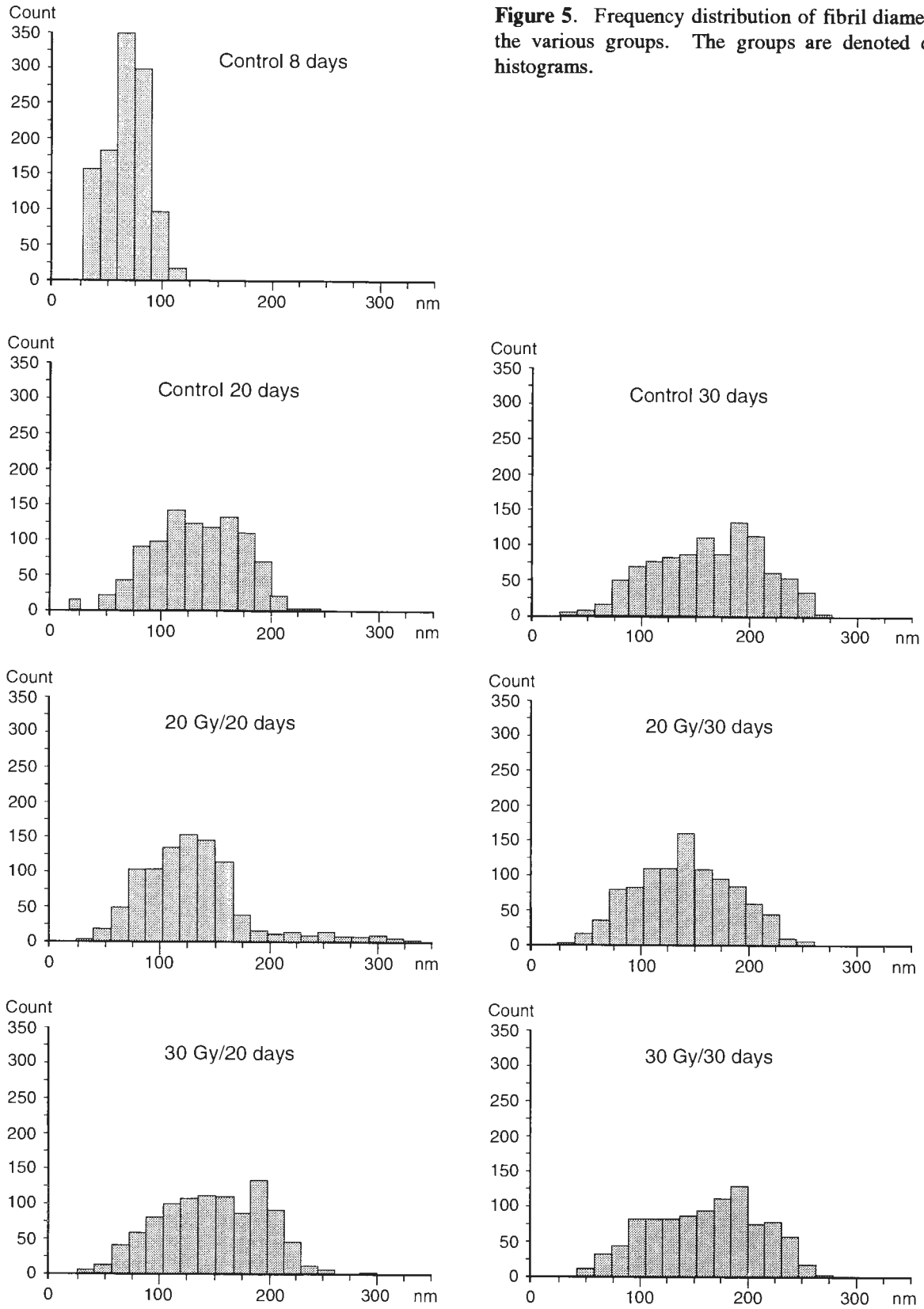


Figure 5. Frequency distribution of fibril diameters in the various groups. The groups are denoted on the histograms.

(n = 42), mean tail length was 10.3 mm ± 1 standard deviation (SD) at birth, and 29.0 mm ± 1.3 SD at 8 days of age. (The slight difference in tail length between the control and irradiated groups on day 8 was deemed an irrelevant chance happening, since until then all groups had been exposed to identical conditions). On day 14 after birth, mean tail length was 51.7 mm ± 2.7 SD in the control group as compared with 39.7 mm ± 4.7 SD in the irradiated groups. On day 20, mean tail length was 60.7 mm ± 0.8 SD in the control group, compared with 50.0 mm ± 1.1 SD (i.e., 17% less) in both irradiated groups (there was no difference between the 20 Gy and 30 Gy groups). On day 30, no appreciable further growth was manifest in the irradiated groups: mean tail length was 52.0 mm ± 1.2 SD in the 20-Gy/30d group and 50.2 mm ± 0.4 SD in the 30-Gy/30d group, compared with 75.2 mm ± 0.6 SD in the control group; the difference between control and irradiated groups increased to 33%. As the two irradiated groups did not differ significantly in mean tail length, it was estimated as a common value.

Tail histology

Figures 2a, 2b and 2c show the histological pictures of the tail on day 30 (longitudinal and crosswise sections). In the control tail (Fig. 2a, with the transverse section enlarged), the skin has a normal stratum corneum (**1**) and stratum lucidum (**2**) the hair follicles (**3**) are clearly seen both on the longitudinal and transverse sections. Normal muscle fibers (**4**) and collagen bundles (**5**) appear distinctly, as do arteries and veins (**6**, **7** in Fig. 2c). The bone structure is developing and the intervertebral discs look normal.

The tail irradiated with 20 Gy (Fig. 2b) manifests changes in the skin: a thinner stratum corneum as compared to the control tail, and no visible stratum lucidum. There are also fewer hair cells. The underlying tissue is edematous. The most striking feature is the underdevelopment of the bone structure and intervertebral discs.

Figure 2c shows a tail irradiated with 30 Gy. On the whole, this tail is larger, which is due to edema. The skin has a heavy stratum corneum due to lack of desquamation. On the longitudinal section, the deleterious effect of radiation on the bone formation is seen. Note the heavy edema especially in the intervertebral disc. Figure 3a, representing a control section, and Figure 3b, representing a 30 Gy-group section, show the enlarged artery and vein (**6** and **7**). As compared to the control artery, the irradiated tail has a thicker wall and the vein has a thinner lining.

Fibroblasts

Controls One fibroblast from each control group (8-day, 20-day and 30-day) is shown in Figures 4a, 4b, and 4c, respectively. All control fibroblasts manifested

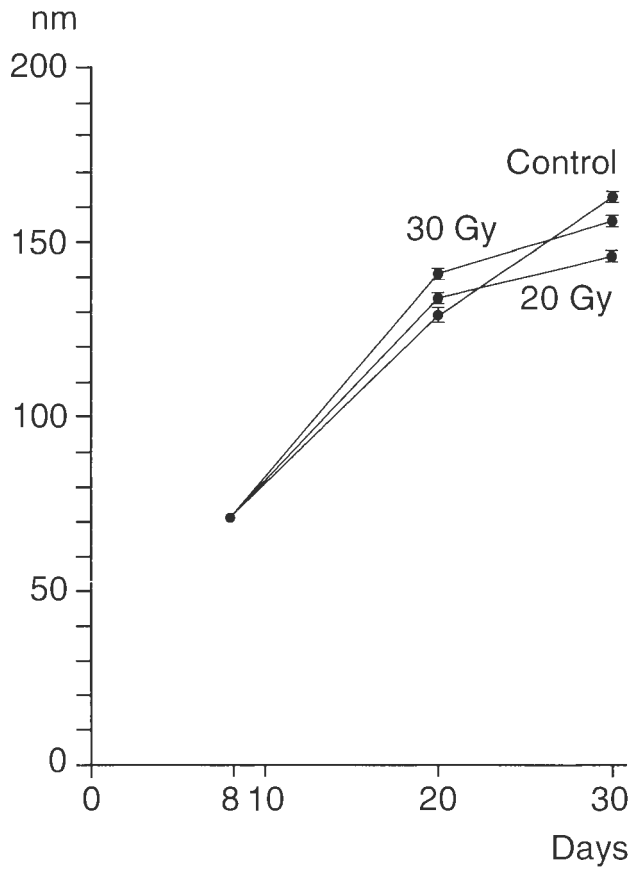


Figure 6. Changes in mean value of fibril diameter over time in the different groups.

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nuclei (N) with normal pores and abundant chromatin predominantly occurring in certain cells. Most nuclei manifested distinct nucleoli. Moreover, in all three control groups, the fibroblasts manifested expanded endoplasmic reticulum (ER) with cisternae (C) and condensing vacuoles (CV), as well as processes extending to neighboring fibroblasts which in the 8-day group were in close proximity to each other, owing to the small fibril diameter (see Figs. 5 and 6). Several cells in control specimens manifested signs of mitosis.

Irradiated groups (Figs. 4d, 4e, 4f and 4g)

Irradiated fibroblasts differed somewhat in appearance from control fibroblasts. The endoplasmic reticulum was not expanded, but was in closely spaced parallel arrangements; the condensing vacuoles were not as abundant as in control tails. Figure 4d shows a cell with scattered chromatin in the nucleus, and Figure 4e shows a definite nucleolus. In Figure 4f, the cell is shown to manifest several putative nucleoli. These findings are of interest from a radiobiological point of view, as it is generally agreed that morphology provides a clue to protein synthesis.

Table 1. Activity of the irradiated and non-irradiated fibroblasts.
(Number of cells in each group as judged from a morphological point of view.)

	8 days		20 days		30 days	
	high	low	high	low	high	low
Control	100	-	100	-	100	-
20 Gy	-	-	96	4	100	-
30 Gy	-	-	47	53	75	25

Accordingly, we analyzed 100 fibroblasts from 5 tails in each group (20 per animal), which were nucleated, and manifested a stellate configuration in the bundles. The fibroblasts were divided into two protein synthesis subgroups, a high-activity and a low-activity subgroup. The morphological criteria for high activity were: abundant endoplasmic reticulum, expanded and with large cisternae and condensing vacuoles. Examples of this are to be seen in Figures 4a, 4b and 4c. Naturally, there were fibroblasts manifesting activity in the intermediate range, but if the criteria were strictly observed, there was no doubt to which subgroup a cell belonged. In Figures 4d and 4e, for example, the fibroblasts differ from controls, but according to the criteria, belong to the high activity subgroup.

As can be seen from Table 1, which shows the distribution of high and low activity fibroblasts, more than half of the cells irradiated with 30 Gy manifested reduced protein synthesis on day 20 (12 days after irradiation), and a quarter of the fibroblasts manifested low activity on day 30.

Fibril diameter

Figure 5 shows the frequency distribution of fibril diameters in controls and irradiated animals (1,000 measurements from 5 animals in each group). Frequency distribution histograms manifested acceptable, normal distribution. Values for the 20-Gy/20d group manifested slight skewness (+1.38), though it should be borne in mind that, in group comparisons, it is the mean values that are of interest.

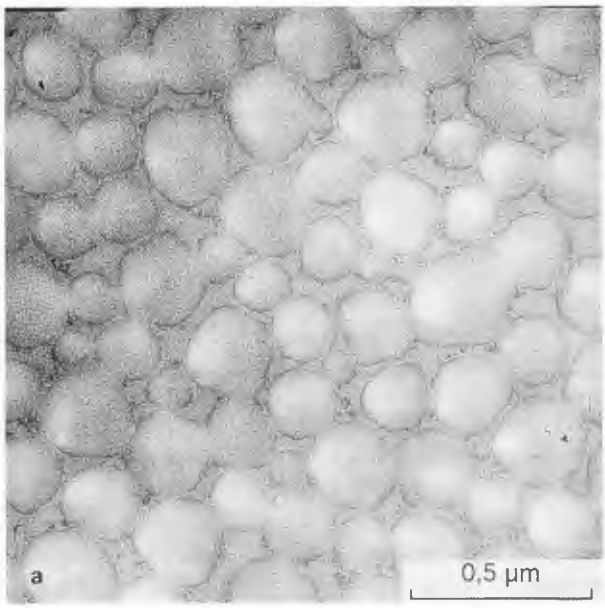
Figure 6 shows the mean fibril diameters for the three groups at 8, 20 and 30 days. In the control group, mean fibril diameter was 71 nm ± 0.5 standard error (SE) at day 8 after birth, 129 nm ± 1.3 SE at day 20, and 163 nm ± 1.5 SE at day 30. In the irradiated animals, mean fibril diameter at day 20 was 135 nm ± 1.7 SD in the 20-Gy group and 141 nm ± 1.3 SE in the 30-Gy group; the corresponding values at day 30 were 146 nm ± 1.4 SE and 156 nm ± 1.5 SE. Thus, at day 20, the increase in mean fibril diameter was greatest in the 30-Gy group, differing significantly from that in the 20-Gy group (141 versus 135 nm, $p = 0.0001$), which in turn differed significantly from that in the control group (135 versus 129, $p = 0.0064$). At day 30 (i.e., 22 days after irradiation), it was the control group that manifested the greatest increase in mean fibril diameter instead, differing significantly from the 30-Gy group (163 versus 156 nm, $p = 0.0027$), which in turn still differed significantly from the 20-Gy subgroup (156 versus 141 nm, $p = 0.0001$).

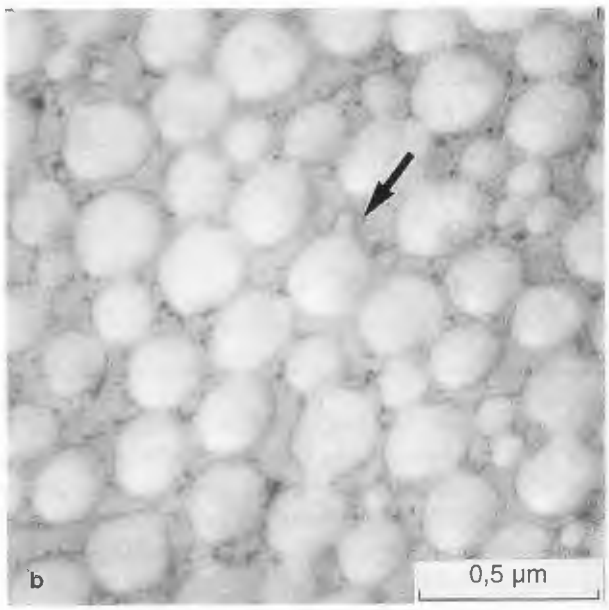
Typical cross-sectional micrographs of control, 20-Gy and 30-Gy group fibrils at day 30 are shown in Figures 7a, 7b and 7c, respectively. A noteworthy manifestation in some fibrils was the appearance of something resembling "budding" (arrow in Fig. 7b). Although the significance of this phenomenon is unclear, it might be a feature of fibril growth.

Band length

Typical longitudinal micrographs showing striation patterns in control, 20 Gy and 30 Gy-group fibril at day 30 are shown in Figures 8a, 8b and 8c, respectively, and corresponding mean group banding periods, in Figure 9. In the 8-day control group ($n = 6$), mean band length was 53.5 nm ± 0.5 SE. In the six animals from each group sacrificed at day 20, mean fibril band length was 57.1 nm ± 0.2 SE in the control group, slightly less (56.8 nm ± 0.3 SE) in the 20-Gy/20d group, and slightly greater (57.7 ± 0.3 SE) in the 30-Gy/20d group. These values did not differ significantly from the control. However, the value of the 20-Gy/20d was significantly lower than that of 30-Gy/20d ($p = 0.04$).

Thus far, the band sequences seemed to follow a normal development in all three groups. At day 30, however, the mean values diverged to 58.0 nm ± 0.2 SE in the control group, 54.8 ± 0.4 SE in the 30-Gy/30d group, and lowest, 52.9 ± 0.1 SE, in the 20-Gy/30d group; the difference was similarly significant in both cases ($p = 0.0001$). No correlation was found between band length and tail length. It was noted that the irradiated tails were manifestly flaccid, though no satisfactory objective method was available to quantify this.





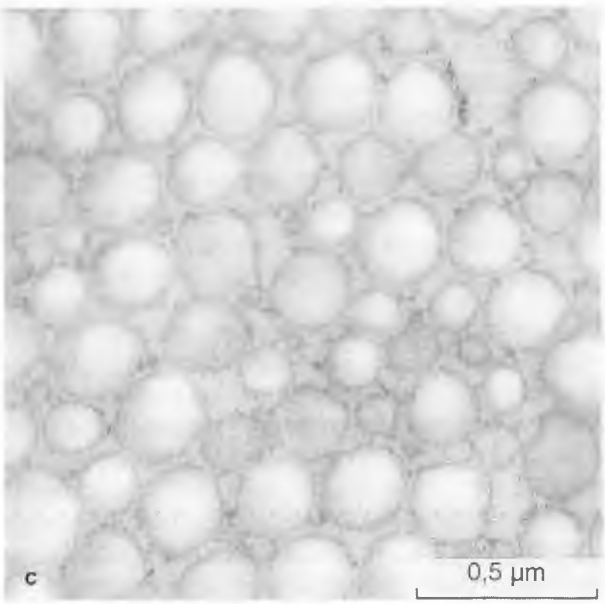


Figure 7. Cross section of collagen fibrils on day 30. (**a**) Control. (**b**) Irradiated (20 Gy) (arrow: "budding"). (**c**) Irradiated (30 Gy). Bars = 0.5 μm.

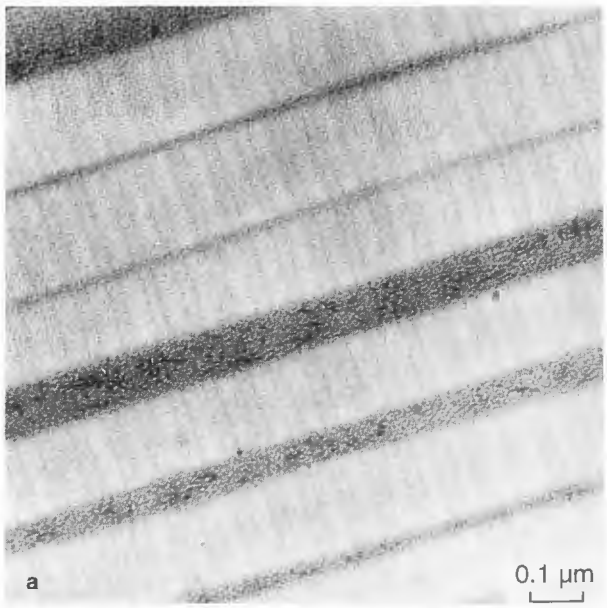
Discussion

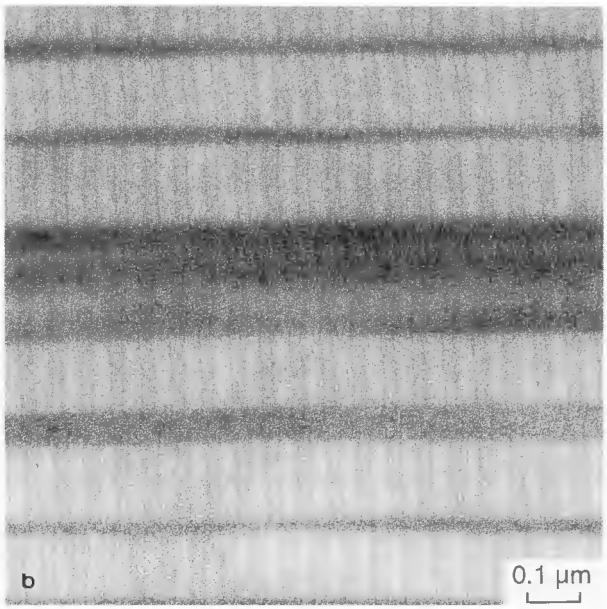
In a tail-stunting experiment similar to that presented here but with a different aim (Jonsson *et al.*, 1985), stunting was found to be as much as 20% after a dose of 30-Gy, which is the same dose as was used in the present experiments. Tail growth retardation in the irradiated groups was also noted 10 days after treatment, as in the present study where paraffin sections from day 30 (Figs. 2a, 2b and 2c), manifested all the classic effects of radiation on tissue: edema with swelling of the tissue, effects on the skin, the hair and on bone formation; the effect was greater in the 30-Gy group than in the 20-Gy group, even if tail length was the same in both groups (Fig. 1). That ionizing radiation exerts effects on vessels, hair and growing tissue has long been known. Its effect on hair was described by Daniel already in 1896, three months after the discovery of X-rays by Roentgen. It was reported to have a growth inhibitory effect in the lower animals by Bohn (1903), and in the higher animals by Perthes (1904); and edema due to a radiation-induced vessel permeability was demonstrated by Miescher (1925). Taken together, these findings explain the retardation of tail growth found in the present study.

The active fibroblast is a highly differentiated cell with a complex protein synthesis, the end product being the collagen fibril. The effect of radiation is manifest both in the cell and fibrils. The well-known edema present after irradiation cannot be evaluated in fibroblasts with the present investigation procedures. On the other hand, it is possible to evaluate the effect of irradiation on protein synthesis. The endoplasmic reticulum largely reflects cellular activity. According to common textbooks of histology (e.g., Fawcett, 1994), "the endoplasmic reticulum is sparse in inactive cells," and "closely spaced in parallel arrays."

In the present study, morphological evaluation of the fibroblasts showed all control cells to manifest a high level of protein synthesis activity.

In the 30-Gy/20d group, i.e., 12 days after irradiation, its effect is reflected in the low level of synthetic





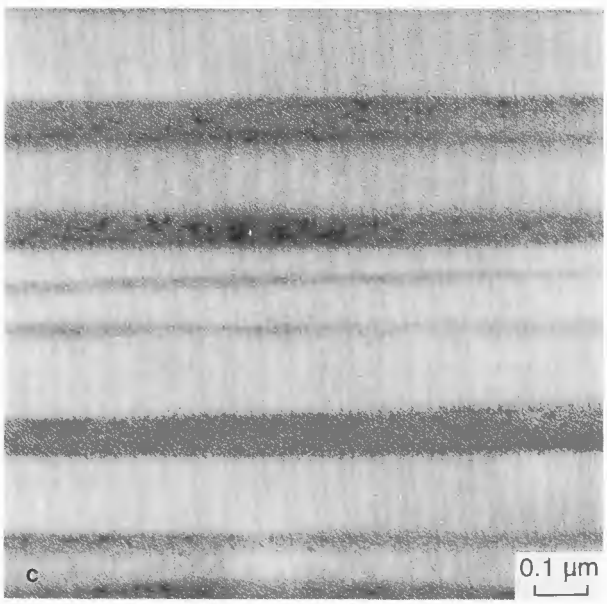


Figure 8. Transmission electron micrographs showing collagen fibrils from three different groups on day 30. (**a**) Control. (**b**) Irradiated (20 Gy). (**c**) Irradiated (30 Gy).

activity manifested by half of the fibroblasts, and the absence of the large cisternae seen in the control groups. In the 30-Gy/30d group, i.e., 22 days after irradiation, comparable findings were made in a quarter of the fibroblasts. Biochemical analysis would be required to provide conclusive evidence of this reduced protein synthesis, deduced here on morphological grounds.

The effect of radiation on fibrils would appear to be twofold. Irradiation of the fibroblast should result in a product (fibril) of smaller diameter. In addition, there is the well-known radiobiologic effect of edema. Collagen is known for its hydropexic capacity. The significantly greater fibril diameter in both irradiated groups at day 20, as compared with 20-day controls, is consistent with the presence of edema. Whether "cross-links" as described by Leontiou *et al.* (1993) and Liu *et al.* (1989) are of importance in the actual experiments cannot be evaluated. The significantly reduced fibril diameter in both irradiated groups at day 30, as compared with 30-day controls, suggests not only that the edema has decreased but also that the effect of irradiation on fibroblasts is now manifest. Again, biochemical analysis would be required to provide conclusive evidence, one way or the other.

The experiments also showed an effect of radiation on collagen banding (Fig. 9). As it is unknown whether there is any difference between the band period at the tail root and that at the tip, all specimens were taken from the mid-part of the tail. In the normal tails, the band period of the fibrils increased with age; the mean value was 53.6 nm at 8 days, increasing to 58.0 at 30 days. According to current textbooks of histology, normal collagen fibrils have a period of about 64.0 nm. Extrapolating from the present findings, the band length would not have passed the 60.0 nm level before the age of 60 days, which is reasonably consistent with the time required for the mouse tail to reach its final length (Jonsson *et al.*, 1985).

Preliminary evaluation of the radiobiological effect on the band period in collagen fibrils may also be based on the histological picture (Figs. 2a, 2b and 2c), where

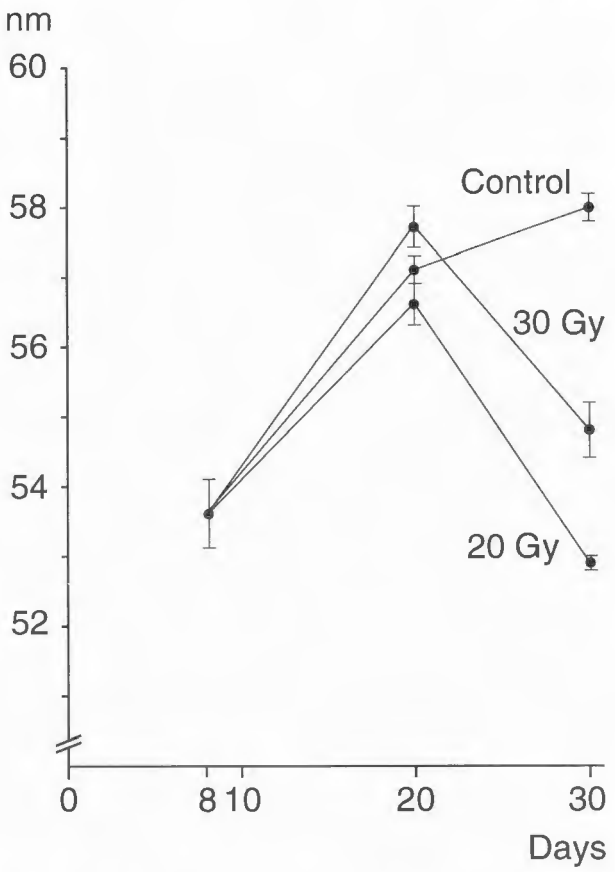


Figure 9. Mean distance between each period along the fibril axis in the different groups at different times.

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edema is seen to surround most of the tissues. The occurrence of edema or increased vessel permeability has recently attracted widespread interest in the field of radiobiology.

In a survey of the physiological response of endothelial cells to ionizing irradiation, Evans *et al.* (1986) and Fajardo (1989) described in detail a number of processes, e.g., "early increase in permeability which is well documented." The occurrence of a biphasic response to doses of 11-18 Gy delivered to the rat thorax was also reported: the first phase occurred on day 1 after irradiation, and the second phase on day 19 (Evans *et al.*, 1986). Thus, edema affecting cells and interstitial tissue would logically be assumed to affect the collagen fibrils due to uptake of water. If so, it can be deduced that swelling (acting in three dimensions) would also result in an increase in band length, the higher the dose, the longer the period. Thus, the band length in tails irradiated with 30-Gy was greater than in those exposed to 20-Gy (both on day 20 and 30, cf. Fig. 9). The considerable diminishing in band length from day 20 to day 30 in the irradiated tail may be explained as follows: the edema must have reached a peak around day 20 (according to general radiobiological knowledge), though some edema was still present on day 30 (Figs. 2b and 2c). Even if the fibroblasts of the dermis constitute a "slowly proliferating tissue as suggested by Denekamp and Fowler (1977), in accordance with the foregoing discussion, the irradiation must have exerted an effect on the fibroblasts and their synthesis of collagen. In one of the previous studies already mentioned, concerning lung fibrosis (McAnulty *et al.*, 1991), both stimulation and degradation of collagen synthesis were observed at day 25 after irradiation, though the difference from baseline values was not significant.

An increase in dermal collagen biosynthesis after ionizing radiation has been reported to occur both in the mouse (Panizzon *et al.*, 1988) and in the rat (Kitagawa *et al.*, 1961), and rat skin (DeLoecker *et al.*, 1976); the experiments were made on adult animals in all three studies. Decrease in the synthesis of collagen after radiation has been reported by Ullrich and Casarett (1977) and Drozdz *et al.* (1982). None of the experiments mentioned above can be compared with the present study in growing mice. In the present experiments, it would seem reasonable to assume that 30 Gy must exert a stronger effect than 20 Gy. Probably, both the fibril diameter and the band length are determined by a balance between the effect of edema on the fibrils and the deleterious effect of irradiation on the fibroblasts.

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Discussion with Reviewers

M. Tzaphlidou: The method that the authors have used for measurements of the collagen fibril band length has no scientific level as this method is not reliable. The measurement of band length by electron microscopy requires micrographs with very high resolution, which the authors do not offer, and much more detailed analysis, which will be able to take into account the ways of specimen preparation for E.M.

Authors: We are at a loss to understand this view. The magnification was optimal, allowing estimation of 50 band periods on the same fibril. This is made possible with the help of a preparation microscope, the periods on the micrographs being marked out with a sharp point, and the overall length of 51 such markings (= 50 periods) measured with a slide caliper. The length in millimeters so obtained is then adjusted according to the microscopy magnification and the micrograph magnification to yield the final measurement results in nanometers. If, on the other hand, we were to perform the magnification as the reviewer seems to be suggesting (which is, of course possible with the JEOL CX 100), we could, e.g., get only five periods to be measured with the same slide caliper, i.e., with 10-fold greater measurement errors as a result. Thus, not only does our method yield measurements that are as optimal as it is possible to obtain with the present level of technology, but it enabled us to identify all the periods belonging to the same fibril. We feel that this is a high enough level of scientific precision for the purposes of the study.

M. Tzaphlidou: Figure 5 does not offer anything and is better omitted. It is better to present mean diameter values in a table.

Authors: We feel the figures show the results better than a table. Actually, the idea of using a histogram showing fibril diameter and frequency was adopted from a paper of which the reviewer herself was a co-author (Leontiou *et al.*, 1993, Fig. 2).

M. Tzaphlidou: What do the authors mean by the appearance in cross-sections of something resembling "budding"?

Authors: Although extremely difficult to describe suc-

cinctly, and impossible (at this stage) to quantify in any of the groups, the phenomenon was nonetheless distinctly manifest, and we believe it to have some significance. However, most readers will have an idea of what "budding" looks like. As it resembled budding, it seemed sensible to describe it as "something resembling 'budding.'" We shall be studying the phenomenon in greater detail later, of course, but feel its occurrence worth recording now.

M. Tzaphlidou: In Figure 8, where are the striation patterns? In type I collagen, there are 12 bands in each period. Here, none can be recognized.
Authors: Here, we believe the reviewer to be referring to type I collagen in adult mice. In these young mice, the pattern is different, and perhaps for that reason the micrographs are of interest inasmuch as they show this difference in pattern. At all events, hitherto, it has not proved possible to show more striation, whatever method was used.

M. Tzaphlidou: The cross-links that are reported by Leontiou *et al.* (1993) and Liu *et al.* (1989), I would expect to influence collagen architecture rather than fibril diameter. But first of all, we should address the question if there is a relationship between findings *in vitro* and *in vivo*, as it is well known that collagen fibrils *in vivo* are also closely associated with extracellular matrix components.
Authors: One of the papers referred to, Liu *et al.* (1989), also listed by us in the references in our paper, contains the following statement: "Examination of irradiated collagen revealed a marked increase in fibril diameter, presumably generated by the interfibrillar cross-links..." Naturally, we have no quarrel with this view, but our conclusions were based on radiobiological findings that edema is the most important factor.

P.B. van Wachem: Is phenomenon of "budding" observed more often in controls, or the other way around, more often in the irradiated group? Please describe precisely.
Authors: Although it was impossible to quantify the occurrence, it was evident that many more such irregularities or "buddings" were present in the younger, non-irradiated animals.

P.B. van Wachem: Do the authors suggest that a 64 nm band length is reached at day 60 of age, i.e., with a full grown tail? Thus far, I myself have just been aware of fibril diameter during growth.
Authors: No, we make no such suggestion. We write "... the band length would not have passed the 60.0 nm level before the age of 60 days..." We hope this answers the reviewer's query.

J.R. Trevithich: What role do you believe free radicals generated by the radiation played in the changes seen, since several of the responses appear to be dose-dependent?
Authors: Although, radiobiologically speaking, free radicals must be generated, it is difficult to interpret their impact on the result.

J.R. Trevithich: Is there any evidence that free radical scavengers could reduce the radiation damage observed?
Authors: Although we have no such evidence, it should be borne in mind that the animals were irradiated at an age of 8 days, when they were still fed by their mother. The local application of anything in the tail would destroy the experiment, of course, and we think it must be very difficult to investigate this issue.