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Studies in Wound Healing: *

IV. Retardation of Contraction by Local X-Irradiation, and Observations Relating to the Origin of Fibroblasts in Repair

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PARTIAL or complete closure of wound defects by inward movement of pre-existing tissues may occur in mammals, including man, to a far greater degree than is generally recognized. Reduction in the area of a surface defect or contraction should be distinguished from gross shortening of scar tissue which may also be described as contraction, or as contracture. Neither process is yet well explained, and there probably is an overlap of mechanisms of both phenomena. However, in the first case the process is principally one of migration of pre-existing tissues and in the second of remodelling of new formed tissues.

Experimental analysis of wound closure by contraction has been made in guinea pigs, with realization of anatomical differences from man. The contraction mechanism has been shown to originate from loose connective tissue proliferation localized beneath the marginal zone of the wound. This new formed tissue rather than the mass of granulation tissue filling the wound appeared to act as the "organ of contraction."[6, 27] New collagen formation seemed to tether the advancing margin but was not itself essential for normal contraction dynamics.[7] Histological study showed marked orientation of fibroblasts in the critical zone of the wound margin.[8] These observations suggested that contraction might be due to massed cellular migration. In continuing studies of the mechanism, open wounds in guinea pigs were subjected to x-irradiation at varying times and effect on contraction measured.

Methods

Full thickness wounds measuring 2 × 2 cm. were made through skin and panniculus carnosus of both sides of the trunks of

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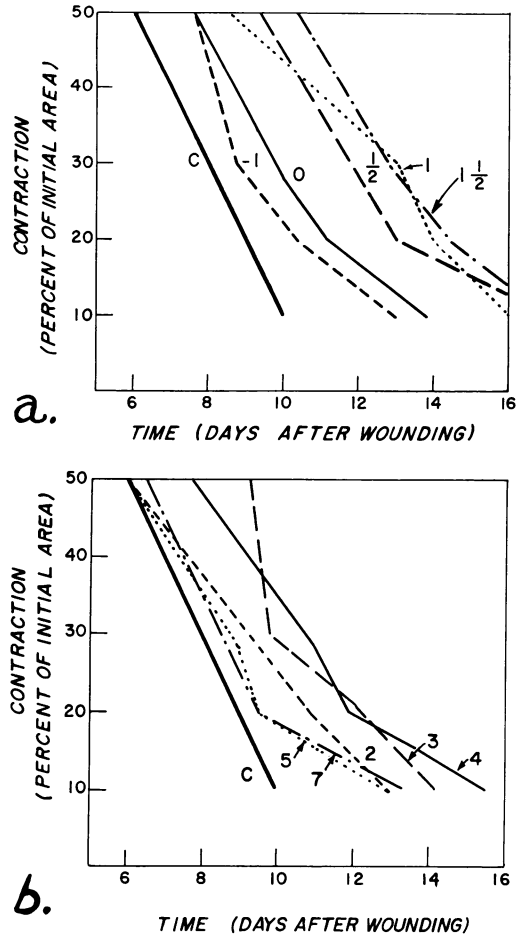

FIG. 1a, b. Contraction measured as percentage of initial area at various times after wounding. Curve "C" represents unirradiated controls. Numerical designation of other curves indicates radiation time in days after wounding. Each curve represents data from 8 animals with one wound on each side.

male guinea pigs weighing about 300 Gm., as described previously.[6] Wounds were not dressed. Wound outlines were traced directly on transparent paper at one- to three-day intervals and areas determined by planimetry.

At varying time intervals before and after wounding, the right sided wound and its periphery were x-irradiated, without anesthesia. Four to eight animals comprised each group. The animals were shielded by collimation and by 0.8 mm. of lead except for a 4 × 4 cm. square portal centered on the wound. Thus a zone extending 1.0 cm. back from the wound edge received radiation. Tubular plastic holders for cephalad and caudad extremities restrained other than respiratory movements during radiation; 750 roentgens of x-irradiation were delivered to the right sided wound (10 MA, 100 KV, 0 filter, at a distance of 20 cm., time 2 min. 5 sec.). The dosage was selected after preliminary experiment showed death occurring in five days following 6,000 r similarly delivered, in eight days after 4,000 r, in nine days after 2,000 r, with survival at doses of 1,000 r and below. The animals receiving 750 r of irradiation lost none or small amounts of weight immediately following either operation or radiation, but regained this promptly and remained healthy, growing normally for a five-week period of follow up. Some animals were listless for a few days. None had diarrhea.

Penetration of radiation was measured as exit dose with a Victoreen ionization chamber at the surface of the wound on the opposite side of the animal, at levels which varied from 80 to 115 r. Control data were derived from a group of unirradiated guinea pigs identically wounded.

Another group of guinea pigs weighing 400 to 600 Gm. had two wounds measuring 1.4 × 1.4 cm., placed on their right sides, the wounds spaced 3.5 cm. apart. The opposite sides were left intact. Four animals were traced daily as controls. Four animals received 750 r centered on their posterior wounds, with shielding that limited radiation to a zone extending 1.0 cm. peripheral to the wound edge, and four received radiation to the anterior wound. All were irradiated 36 hours after wounding.

An additional group of animals was similarly wounded, irradiated and sacrificed at intervals for histological study. Tissues were fixed in Bouin's fluid and sections stained with hematoxylin and eosin. Terminal perfusion of the vascular system with

a carbon suspension of uniform particle size (Pelikan No. C 11/1431 a.)[10] was done in unirradiated and irradiated animals in order to study distribution of capillaries.

Results

Contraction Measurements. Contraction was retarded by radiation to a degree which varied with the time of radiation with respect to time of wounding. In general, radiation resulted in a lag in initiation of the rapid phase of contraction. In cases where maximal effects were observed the process proceeded at a diminished rate. Completion of contraction was delayed but with the 750 r dose used all wounds ultimately closed fully.

Maximum retardation of contraction, in initiation, rate and duration of effect followed radiation between 24 and 48 hours after wounding, with peak effect at 36 hours. Relatively smaller effects were found when radiation was done 24 hours prior to wounding or when radiation was delayed until the fifth to seventh day after wounding (Fig. 1a, b, 2). The greatest

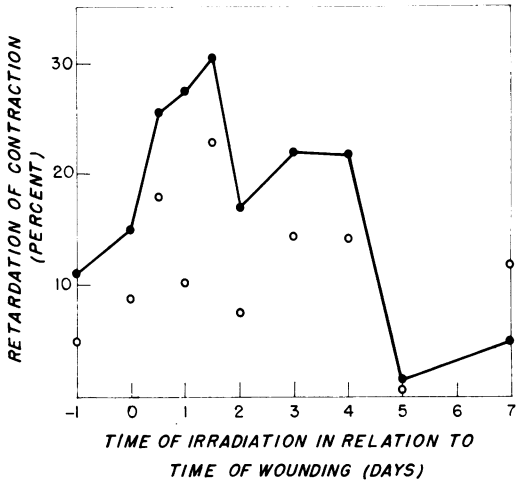

FIG. 2. Variation in total retardation of contraction between the fifth and 21st days after wounding as percentage delay compared with unirradiated controls for various times of irradiation before or after wounding. Open circles indicate wounds on the side opposite those irradiated directly. Each point represents the average of 8 wounds.

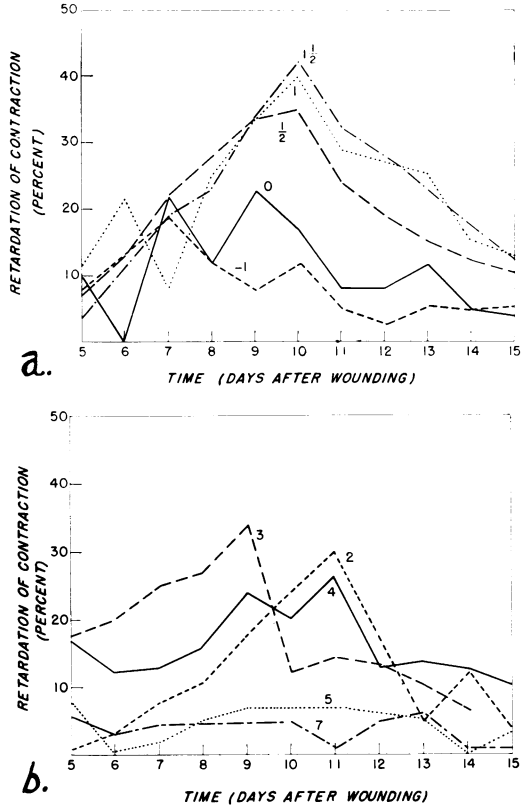

FIG. 3a, b. Retardation of contraction in irradiated wounds as percentage delay compared with unirradiated controls at various times. Numerical designation of the curves are the radiation time in days before or after wounding. Data from 8 animals with one wound on each side.

slowing of contraction when compared with the normal pattern was observed between the ninth and eleventh days (Fig. 3a, b).

Contraction of wounds on the side opposite to those directly irradiated was also delayed. The effects observed were qualitatively similar to those in directly irradiated wounds, but of smaller magnitude (Fig. 2). Doses of ionizing radiation measured at the surface of these wounds were one tenth to one seventh of those administered on the opposite side.

When two wounds were located on the same side of the animal so that complete shielding of one wound was possible, no retardation of contraction occurred in the

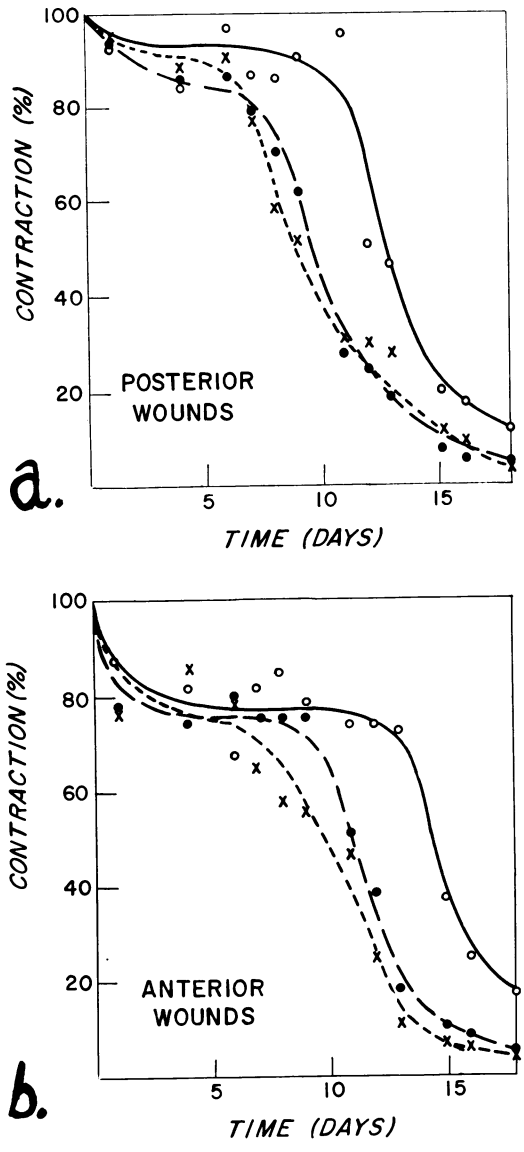

FIG. 4a, b. Contraction as percentage of initial wound area with time in days of: a) anterior wounds in unirradiated animals, anterior wounds in animals where the posterior wound was irradiated and anterior irradiated wounds; b) posterior wounds in unirradiated animals, posterior wounds in animals where the anterior wound was irradiated, and posterior irradiated wounds. Anterior and posterior wounds are separately plotted to control variations in contraction pattern due to regional anatomical stresses. Each point represents mean of data from 4 wounds. Wounds in unirradiated animals are indicated by black dots and broken line curve. Unirradiated wounds in animals having an irradiated wound are represented by crosses and dotted lines. Irradiated wounds are designated by open circles and solid line curve.

shielded wounds (Fig. 4a, b). Retardation of contraction of the irradiated wounds was similar to retardation seen in the first series irradiated at 36 hours.

Histological Observations. The general microscopic effects of local irradiation on new forming connective tissue at the wound margin were similar to those described by previous authors[12] and these aspects do not require detailed description. Subsequent effects were most dramatic when radiation was given at the sensitive period of 24 to 48 hours. Fewer mononuclear cells appeared in the early stages and fewer defined fibroblasts later. The number of polymorphonuclear leukocytes at the wound surface was not reduced, and in some instances appeared to be increased. By the fifth and seventh days in wounds radiated at 24 hours, the fibroblast population was much lower than normal, the fibroblasts were irregular in size, shape, staining and orientation. Some cells were greatly enlarged, some were small and dark staining, some were irregular in outline. There were fewer spindle shaped cells at seven days. The usual oriented pattern was greatly disturbed. There was little collagen fiber formation evident, and the apparently edematous region of repair contained irregular fibrinoid strands (Fig. 5, 6).

Marked inhibition of capillary proliferation following radiation was demonstrated by carbon injection. Dilatation and prominence of probably pre-existing capillaries in the wound base was noted. Reduction in number of capillaries in the new forming tissue was real and not apparent, due to edema, since the total volume of regenerate was no greater and often was smaller than unirradiated tissue at any stage (Fig. 5a, e; 6a, e). Examination of wounds on the opposite side of the animal from the directly irradiated wound showed lesser alterations of morphology (Fig. 5c, d; 6c, d).

Radiation at later periods resulted in lesser effects. Thus radiation of the al-

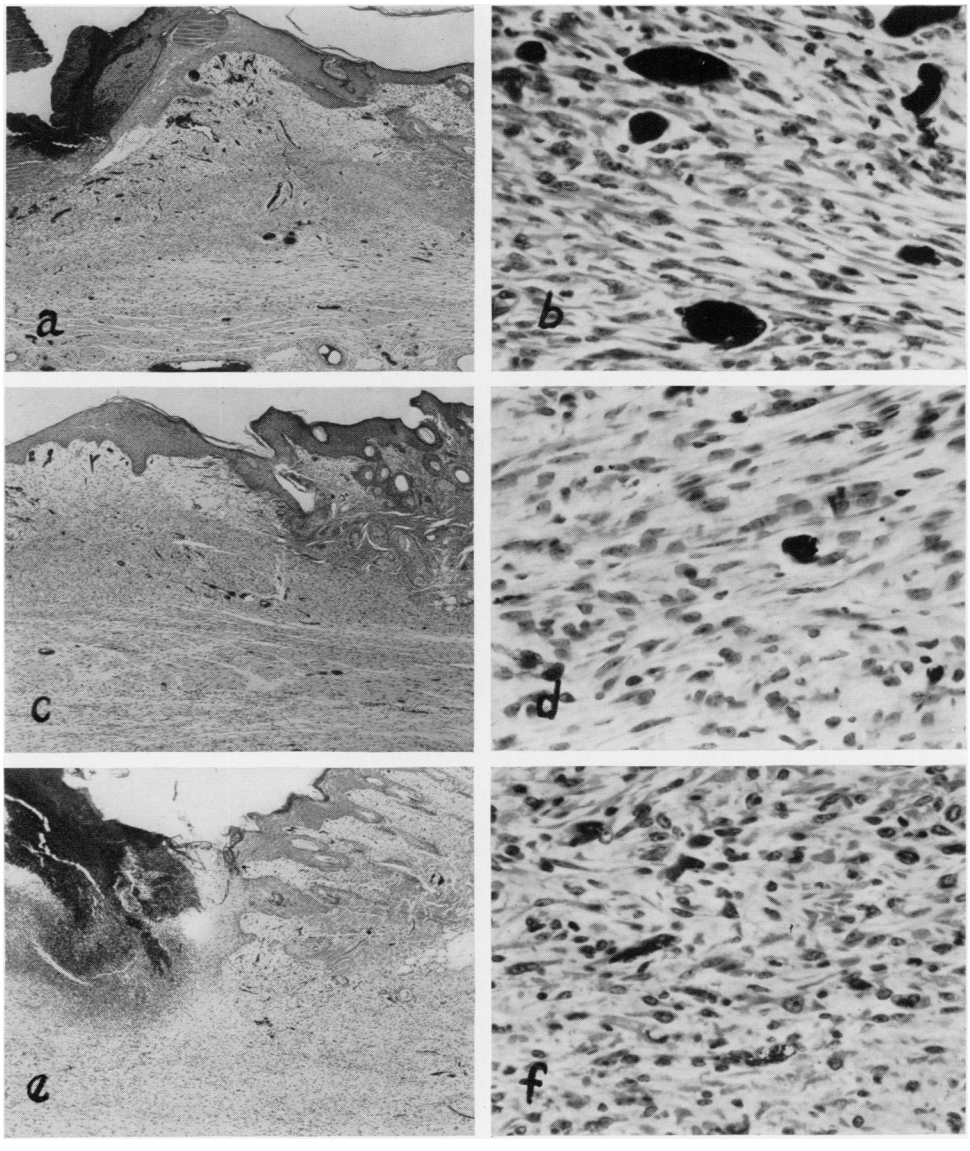

FIG. 5. Photomicrographs of wound margins at 5 days. (H and E; a, c, e are magnified 25×, b, d, f 225×.) At low magnification dermis and overlying epidermis are at the top right, muscle of the abdominal wall along the bottom of the photomicrograph. The remaining tissue is chiefly new forming repair tissue with some residual loose connective tissue overlying the deep fascia and in some cases beneath the dermal edge at the far right. Capillaries injected with carbon suspension appear black. High magnification photomicrographs are representative areas of new formed tissue. Irradiation was done 24 hours after wounding. a and b, Unirradiated wound. Note profusion of new capillaries, and dense cellularity. c and d, Wound on side opposite to directly irradiated wound, receiving only radiation which had penetrated through the animal. Capillaries are reduced in number, cell population is lower. e and f, Directly irradiated wound (750r). There are few new capillaries, the endothelial cells appear to be swollen. Prominent capillaries in the wound base seem to be largely residual vessels in the original loose connective tissue. Fibroblasts are reduced in number, vary greatly in size and shape, and show bizarre forms. Intercellular spaces appear widened and filled with material having fibrinoid appearance. Little organization is evident. Total amount of new tissue is decreased slightly. The epithelium is little affected morphologically.

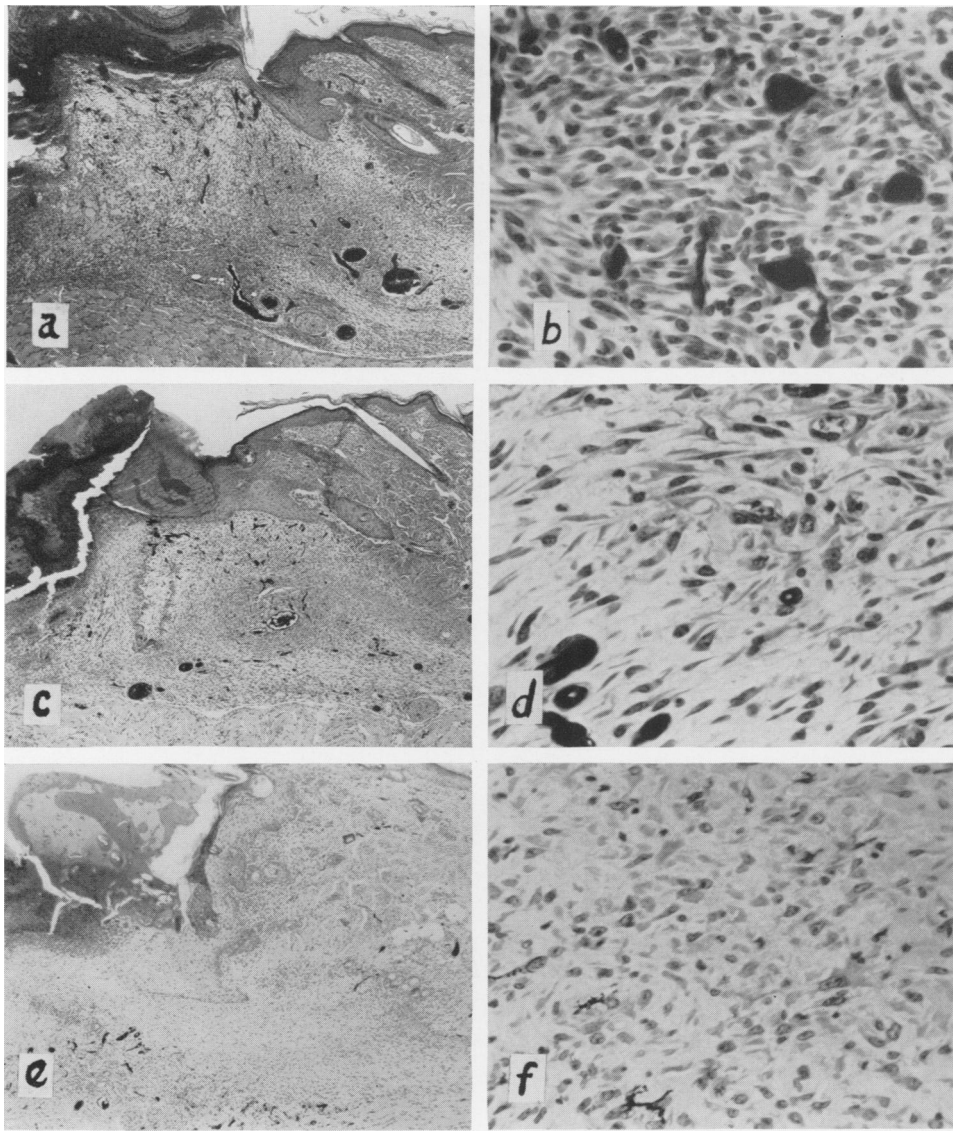

FIG. 6. Sequences similar to Figure 5, at seven days. Irradiation was 24 hours after wounding. a and b, Unirradiated wound. The tissue is well populated with fibroblasts which are more homogeneous in morphology and show orientation. Fibers are assuming prominence. New capillaries are seen in large number. c and d, Wound receiving low radiation dosage. The new formed tissue is somewhat less cellular; the tissue is less dense; capillaries are fewer. e and f, Irradiated wound. Cell morphology is variable and bizarre; little orientation is evident; intercellular spaces show few well-defined fibers.

ready established repair tissue mass at five days or at seven days resulted in markedly less cell change, less edema, and less reduction in fiber content and total amount of new tissue by day 12 when compared with 12-day wounds irradiated early. Irradiated and unirradiated wounds were indistinguishable by 30 days.

Discussion

The present study showed that moderate doses of local radiation administered within

a critical time period after wounding significantly delayed the gross process of contraction, without inhibiting its eventual completion. The total retarding effect increased as the same radiation dose was administered from 12 hours to a peak effect at 36 hours after wounding, then fell. Without radiation, contraction usually became brisk at five days and was well established in its rapid phase at seven days. When radiation was applied after contraction had achieved its momentum relatively little delay resulted. The mechanism apparently had become well established by this time. If the contraction mechanism is basically of cellular origin, as previous studies have suggested,[6, 7, 27] it may depend upon proliferation of a sufficient number of fibroblasts before the phase of rapid contraction, differentiation of some necessary characteristics of fibroblast maturity, or establishment of a satisfactory capillary supply.

Since the radiation beam had no added filtration it can be assumed that most of the energy was absorbed in the skin edge layers and the exposed subcutaneous tissues of the wound opening. However, systemic effect of this radiation must be assumed in view of the penetration measured on the opposite side of the animal. The qualitatively similar but quantitatively different effect on wounds on each side of the animal suggested a marked local effect. This was substantiated by the histological findings described. Failure of radiation to inhibit a completely shielded wound in an animal where the irradiated wound was affected indicated that the effect described was largely a local one at the dosage used.

In the present state of uncertainty about the origin of fibroblasts, about interrelationships of the two principal continuing cell populations in repair—fibroblasts and capillary endothelial cells, and about the specific nature of radiation effects upon these and other components of new forming tissue, precise interpretation of these results in regard to the mechanism of contraction becomes speculative. Maximow[12] early demonstrated that radiation (with doses calculated by Ritchie[19] to be about 450 r) of new tissue forming in celloidin blocks 24 hours after implantation, and at other intervals, resulted in diminution of the number of fibroblasts, absence of mitotic figures, distortion of fibroblast forms, and swelling and nuclear fragmentation of capillary endothelial cells. He speculated on the special sensitivity of fibroblasts about to divide. Sensitivity of fibroblasts to radiation has been shown *in vitro*.[9, 17] It is a classical principle of radiobiology that rapidly dividing cells manifest radiation damage sooner than reproductively less active cells. The decreasing total effect on retardation after three days with increasing time of radiation after wounding might suggest that irradiation injured chiefly that portion of the cell population which was in a sensitive mitotic or differentiating phase at any given time. Maximum total effect at 36 hours might result from the fact that a large part of the population was in a sensitive state at the time of radiation, since cell proliferation was then well under way. Embryological studies indicate increased sensitivity of cells just prior to and during the phase of differentiation.[4, 5, 20] If collagen fiber production may be considered as an index of functional differentiation of the fibroblast, the phase of differentiation would bracket the observed period of maximal radiation sensitivity.

A primary local effect on new capillary formation must also be considered. Stearns early demonstrated by direct observation the close interrelationship of fibroblasts and budding nutrient capillaries in repair.[21, 22] Fibroblasts were never observed far from the advancing capillary bed. Also utilizing a direct vision chamber in the rabbit's ear, van den Brenk found no inhibition of growth of a regenerate after single irradiation with doses below 1,500 to 2,000 r, immediate inhibition with doses

over 2,000 r with resumption of growth at a reduced rate after a variable latent period. At doses from 300 r to 1,500 r capillary bed formation was often modified or destroyed; above 1,500 r, more marked destruction occurred, notably in newly formed capillaries. He believed that a viable connective tissue remnant continued to develop and acted as a barrier to the inhibited vascular component, unless secondary trauma occurred.[24] In these experiments, radiation was given from seven to ten days after appearance of the blastema on the direct vision table. The same author also pointed out that the intensity of damage to capillaries was maximal in the most immature sites, despite lack of clearly demonstrable mitotic figures.[25]

No reports of systematic study of wound contraction following local radiation have come to our attention. Van den Brenk stated that local irradiation with doses as low as 500 r after wounding in the rabbit decreased contraction rate. The effect was dose dependent.[23] Walter and Slome noted that locally applied x-ray up to 1,500 r delayed contraction of circular excised wounds in rats.[26] Radakovich, Dutton and Schilling found no retardation of wound contraction in rats when total body irradiation from 150 to 650 r was given from eight weeks to immediately prior to wounding.[18]

Numerous investigators have described the effect of radiation on repair tissue. Since the forces for contraction have been shown to originate in the new tissues arising from the loose connective tissue, certain of these studies bear on the phenomena reported here. Pohle, Ritchie and Wright found that irradiation of rat skin with 1,000 r prior to incision had no histological effect on healing, while postoperative radiation immediately, at 24 hours and at 48 hours resulted in retardation of healing with maximal effect at 24 hours.[14] Lack of fibroblastic growth, atypical fibroblast giant cells and persistence of fibrin networks were noted. Less effect was noted on epithelium.[15] Low doses did not stimulate healing.[16]

Nathanson studied the effects of beta radiation on incisional wound healing in dogs using histological criteria of healing. He observed accelerated healing with small doses (50–100 r) and retardation with high doses (1,000 r) when radiation was applied immediately after incision. All doses of radiation caused wound retardation when administered 24 hours after wounding, the amount of retardation varying with dose. When wounds were subjected to radiation at 48 hours, retardation was observed with higher doses and no effect with smaller doses.[13]

The histological observations reported here showed depression of fibroblast formation, alteration of fibroblast morphology and inhibition of capillary proliferation, without indication of a priority of effect on either cellular element. Disorganization was also prominent. Distinction between the roles of fibroblasts and capillaries in furthering the movements of contraction was not possible and may well not be separable. The statement that contraction was hindered when ordered marginal proliferation of fibroblasts and capillaries was inhibited therefore seems justified. The depression of fibrogenetic function also noted was probably not critical in the light of previous evidence demonstrating normal contraction in scurvy.[7] These findings were consistent with a theory of contraction invoking directed cell migration at the wound margin.

In the course of studies on the origin of fibroblasts Allgöwer gave total body irradiation of 1,300–1,400 r (x-rays) to rabbits with one ear shielded, and wounds were applied immediately to both protected and unprotected ears. At this high dose level of total body radiation there was depression in granulation tissue formation in both protected and unprotected ears by 50 to 70 per cent. Similar irradiation four days after wounding with wounds protected did

not impede granulation tissue formation. Allgöwer interpreted these results to indicate a systemic effect on a distant source of fibroblasts or their precursors, with acquisition of local wound autonomy by four days.[1] In support of this thesis he has reported production of hydroxyproline containing materal by cells cultured from the blood.[2] MacDonald has recently presented radioautographic evidence favoring local origin of fibroblasts.[11]

The present data demonstrating later qualitative and quantitative effects upon fibroblasts occurring in areas radiated immediately after wounding and at early intervals after wounding strongly suggested local origin of these cells. This was further supported by observation of a retarding effect resulting from local irradiation 24 hours prior to wounding. It seems unlikely that the phenomena reported could have resulted from radiation effects upon extra-cellular elements secondarily affecting cells recruited from blood-borne precursors, particularly when radiation was administered in the earliest post-wounding intervals, when little inter-cellular substance had yet been formed. There was no histological evidence of continued increase in fibroblasts adjacent to the non-proliferating capillaries to indicate a possibility of blockage of vascular recruitment by the mechanism of radiation inhibition of capillary growth. Dependence of mammalian repair upon local cellular response at the site of injury would seem to parallel the local nature of regenerative response in lower animals, as Butler and O'Brien demonstrated in studies of the effect of local irradiation in regeneration of the urodele limb.[3]

Summary and Conclusions

1. Initiation and rate of skin wound closure by contraction in guinea pigs were retarded by moderate doses of local x-irradiation (750 r). Complete closure ultimately occurred.

2. Maximum effect followed irradiation between 24 and 48 hours after wounding; only slight effect followed radiation at five days or later.

3. Depression of fibroblast formation, inhibition of capillary proliferation and disorganization of the architecture of repair resulted from irradiation, concomitantly with retardation of contraction.

4. The data are consistent with a theory that wound closure by contraction may result from oriented cell migration.

5. The findings support the probability of local origin of fibroblasts in wound repair.

Acknowledgment

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Addendum

Additional studies were carried out to define better the radiation dose distribution in the experimental animals. A recently developed in-phantom radiation detector[1a] was made to simulate the irradiated anatomy. This dosimeter gave a three dimensional picture of the relative dose in various parts of the guinea pig and demonstrated that the radiation exerted most of its effects in the first few millimeters of tissue.[2a] From these studies it can be concluded that any systemic effects probably were minimal.

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