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Altered Ovarian Regulation of Wound Healing during Aging (41017)

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Abstract. The healing rate of full-thickness skin lesions is slower in senescent female rats (22–23 months) than in their young adult (5–6 months) counterparts. Fifty percent wound closure times were 9.46 ± 0.31 and 6.45 ± 0.40 days, respectively ($P < 0.001$). Ovariectomy significantly slows the healing process in the mature group but not in the senescent animals. Treatment of ovariectomized rats with low-dose estradiol (0.3 μg/kg body wt) restores the healing rate to nearly normal in mature rats and accelerates the healing rate relative to sham-operated controls in senescent rats. Thus, decreased healing rates in senescent female rats may be due in part to reduced estrogenic potentiation of healing.

The aging process affects systems that require cell division (1, 2) and alters the regulation of cell division by hormones and other physical and chemical stimuli (3, 4). Wound healing is a complex process in which cell proliferation plays an important role (5), and a number of studies involving induction of cell proliferation, accurate remodeling, and termination of new growth suggest that hormones may help to regulate wound healing (6–12). These studies have shown estrogenic, progestenic, and androgenic potentiation of cutaneous healing rates with increased production of fibrinous substances and dilation of local blood vessels (6–9). Progesterone also accelerates vascularization (10), while thyroid hormones increase the rates of cellular proliferation and anabolic processes (11). In contrast, glucocorticoids are reported to reduce the number of cells in DNA synthesis and the postwounding thickness of newly formed epithelium (12).

Reduced rates of wound closure have been reported in older rats (13–15) and humans (15, 16), but these studies did not consider individuals beyond the midpoint of the lifespan (40-year-old humans, retired breeder rats). Experiments employing 21- and 22-month-old rats showed an age-dependent increase in the time required to initiate mitotic activity following experimental myocardial infarction (5), and a slower development of the exudative and proliferative phases of cutaneous wounds (17), actual quantitation of healing per se was not attempted.

The present study, therefore, had three objectives: (a) to compare the healing rate of full-thickness cutaneous wounds of 5- to 6-month- (young adult) and 22- to 23-month-old (senescent) female Wistar rats, (b) to determine whether estrogens play a role in regulating such wound healing, and (c) to determine whether altered estrogenic regulation of wound healing is related to changes in wound healing during senescence.

Materials and Methods. *Animals.* Female, virgin Wistar rats, aged 5–6 (young adult) and 22–23 months (senescent), were obtained from the colony of the Gerontology Research Center, NIA (NIH), Baltimore, Maryland. The mean lifespan for females of this colony is 22.5 months. Body weights are 250–300 and 400–500 g for the two age groups, respectively. All animals appeared to be in good health in so far as there were no observable tumors, respiratory problems, or other maladies except senile cataracts. These occurred in about half of the senescent rats. There were no deaths in the young adult group during the experiments, while about 20% of the senescent group died over the same period.

The reproductive status of the senescent rats as followed for 15 days by sequential vaginal smears was as follows; 55% were in a state of persistent diestrus, 25% shifted from persistant diestrus to metestrus and

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back, with no more than 2–3 days of cornification at a time, 15% were in persistent metestrus with an occasional day (1 in 10–12) of apparent full estrus, 5% demonstrated a single abortive cycle (no full estrus) and then returned to a prolonged diestrus state.

Animals were maintained on the NIH Purina chow diet *ad libitum*, singly housed, and were kept on an 0600- to 1800-hr lighting schedule. Drinking water contained amoxicillin in an amount allowing intake of 20 mg/kg body wt/day based on drinking volume. Some of the rats were ovariectomized (ovx) under general anesthesia with 2,2,2-tribromo ethanol. Ovaries were exposed through bilateral incisions in the anterior axillary line. Wounds were closed with wound clips. Control rats were sham operated (ovaries exposed but not removed). All rats were then returned to their cages for at least 1 week prior to further treatment.

Infliction of wounds. Animals were anesthetized with 2,2,2-tribromo-ethanol. An area approximately 6 × 6 cm on the back lateral to the spine and away from the ovariectomy site was closely shaved with electric clippers. A uniform square, 2 cm on a side was traced with a clear flexible plastic template and a permanent thin line-marker. Incisions were made just within the traced square, deep enough to enter the fascial plane below the subcutaneous fat and full-thickness skin was excised. Almost no bleeding was encountered. Sterile water-soluble lubricating jelly (K-Y) was then applied.

Wound size was measured daily by recording the lengths of each side and calculating the area. As the senescent rats were near the end of their lifespan and final closure appeared to be greatly delayed in some, a 50% reduction in wound area was used to index the rate of wound healing in some of the analyses. A single investigator measured all wounds and was blinded to the treatment dosages which were given by another investigator. Rats were identified by code only. After each measurement sterile K-Y jelly was applied to the wound surface to minimize scabbing.

An attempt was made in early experiments to separate contraction of the wound from epithelialization of the wound. Permanent ink marks were placed near the edge of the wound in young adult rats and the area within the ink marks was compared to the area of the residual wound. There was frequently a decrease in the area encompassed by the ink marks proportional to the decrease in the area of the wound itself. The later experiments using aged rats are based only on wound measurements and no further efforts were made to separate contraction from epithelialization although both are likely to be components of the overall process.

Administration of estrogen. In order to assess the role of estrogen in wound healing, groups of both young and old ovariectomized and sham-operated rats were treated with various doses of 17β-ethinylestradiol(E_2) in peanut oil injected subcutaneously beginning on the day of wounding and continuing throughout the 2- to 3-week period of wound healing evaluation. Both sham-operated and ovx controls received peanut oil vehicle in similar volumes to the treated rats.

Histology. At the conclusion of several experiments an area encompassing the residual wound with normal appearing surrounding skin was excised, fixed in 5% buffered Formalin, and prepared for light microscopy. The residual wound size was measured between epithelial edges and from base to surface with a calibrated micrometer. Overgrowth of the wound crater by thin, newly formed epithelial sheets was recorded. Inflammatory cells in the crust overlying the wound, in the tissue within and below the wound and granulation tissue, characterized by newly vascularized tissue, was semiquantitated on a 0 to 4+ scale. 0 represents none and 4+ represents the most dense amounts. This evaluation was performed via a single-blind protocol in that identities of samples were not revealed to the microscopist until completion of the procedure.

Assessment of circulating white blood cell counts. Blood obtained from tail veins or cardiac puncture was anticoagulated with a small amount of heparin and white cell counts were obtained by a standard Coulter counter procedure in the Baltimore City Hospital's laboratory.

Results. *Effect of age on the rate of wound healing.* Figure 1 shows a comparison of the time courses of full-thickness cutaneous wound healing in a typical experiment with young adult and senescent female Wistar rats. Generally, old animals exhibit an initial lag period with slow rate of closure. In contrast young animals show an early phase of rapid closure (1 to 2 days), after which a slower rate continues for 9–10 days. After approximately Day 5 in old rats there is an increased rate of healing which is roughly comparable to that of younger animals. (Initial slopes: young = 15.5 ± 1.4%/day; senescent = 4.3 ± 0.3%/day, $P < 0.001$. Later slopes: young = 4.3 ± 0.3%/day; senescent = 6.1 ± 0.5%/day, $P < 0.005$. All points for the younger animals are significantly different from the corresponding points for the old animals ($P < 0.01$). However, some variability exists with respect to the relative initial and later healing rates in both age groups. For this reason it was decided to use 50% healing times as an index of the extent of wound healing.

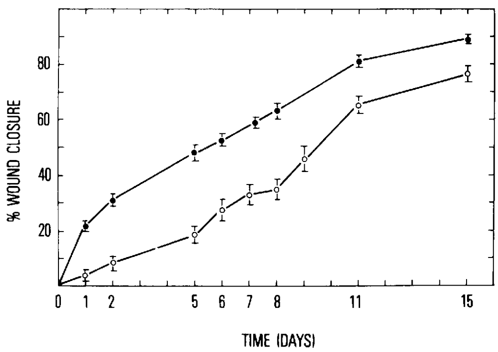

FIG. 1. Time courses of wound healing in young adult and senescent rats. Rats were lesioned and assayed for wound healing as described under Materials and Methods. ●, Five- to six-month-old rats; ○, 22- to 23-month-old rats. Values represent means ± SE's for eight mature and five senescent animals.

Figure 2 compares the 50% healing times for 19 young adult (5–6 months) and 12 senescent (22–23 months) animals examined in four separate experiments over a 5-month period. Although some variability exists with respect to absolute healing times from experiment to experiment, it is obvious that the younger rats heal faster than their older counterparts. No senescent rat reached 50% wound closure in less than 8 days, while only 2 of 18 mature rats took longer than 8 days (both = 9 days) to reach this point. Age differences are significant at the following levels for the four experiments, respectively: $P < 0.05$, $P < 0.02$, $P < 0.001$, and $P > 0.05$ (n.s.). Overall the age differences are significant to $P < 0.001$.

Granulation tissue thickness measured in fixed stained paraffin sections tended to be greater in mature animals regardless of

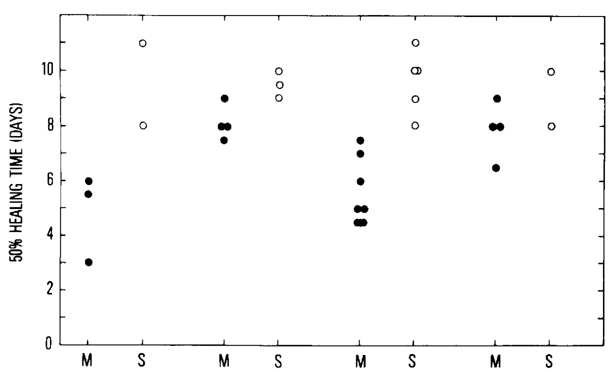

FIG. 2. Fifty percent healing times for mature and senescent rats in four separate experiments. Analysis of wound healing was performed as for Fig. 1 in four separate experiments over a 5-month period. Individual 50% healing times are shown for mature, 5- to 6-month-old rats, ●; and senescent 22- to 23-month-old rats, ○.

treatment than in senescent animals, even when corrected for the percentage healing. However, due to large variation differences were not significant to $P < 0.05$ (Table I). There was no difference in inflammatory cell infiltration in any of the groups. Finally, white blood cell counts obtained from blood in a random sampling of animals gave values of 3687 ± 519/mm^3 and 2450 ± 464/mm^3 for eight mature and six senescent animals. These included sham-operated, ovariectomized and estrogen-treated animals, which were indistinguishable from each other. The difference in means is not statistically significant, nor do the means differ from unwounded rats (range 2000–4000/mm^3).

Effect of ovariectomy and estrogen on wound healing in mature and senescent rats. Since estrogens have been reported to potentiate wound healing (6, 7) the age differences observed here could in part be due to the well-known abnormal ovarian function (see Animals under Materials and Methods) and diminished estrogen levels in older rats (18, 19) and possibly also to decreased sensitivity of healing tissues to estrogen. Before testing this possibility it was necessary to determine the effects of E_2 on wound healing in young adult ovariectomized rats. Figure 3 compares the effect of various E_2 doses on the time required for 50% wound healing in 5- to 6-month-old ovx animals in six separate experiments using one or two doses per experiment. Since some interexperiment variability exists with respect to healing times in ovx as well as intact animals, data are expressed in terms of absolute differences compared with ovx untreated rats. Relatively low E_2 doses (0.33 and 1 μg/kg body wt) administered daily over the course of the experiment reduced 50% healing time by about 1 day over controls, ($P < 0.008$ and 0.05, respectively) while higher doses (3.3 and 10 μg/kg body wt) cause no reduction and may actually prolong healing time by about 0.7 days ($P > 0.05$, not statistically significant due to large variation). The higher E_2 doses appear to slow the rate of wound healing in senescent rats (22–23 months) by 1–2 days (data not shown). For this reason, it was decided to examine the effects of age on estrogen potentiation of wound healing using 0.33 μg of E_2/kg body wt daily.

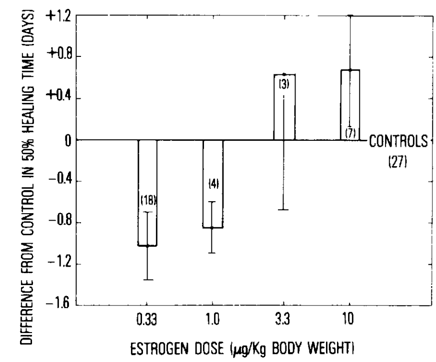

FIG. 3. Effect of various estrogen dosages on time required for 50% wound healing in ovariectomized mature rats. Ovariectomy, estrogen administration, lesioning, and assessment of wound healing were as described under Materials and Methods. Bars represent standard errors for the numbers of animals indicated in parentheses.

Young adult and senescent rats were each divided into three experimental groups; sham operated vehicle, ovx vehicle, and ovariectomized E_2 treated. Five separate experiments were carried out over a 6-month period. Fifty percent healing times were determined for each group in each experiment. Table II shows the results of these experiments. Values are the 50%

TABLE I. HISTOLOGIC FEATURES OF WOUND SITES AFTER 2 WEEKS[a]

	Young adult (19)[b]	Senescent (7)[b]
Granulation tissue	2.2 ± 1.0	1.3 ± 1.1
Polys in crust	1.8 ± 0.7	1.7 ± 0.7
Polys in wound	1.5 ± 0.9	1.6 ± 0.7

[a] A scale of 0–4 with 0 being absent and 4 being dense was employed. Observations were repeated by the same investigator on three separate occasions and a single figure used for each animal.
[b] Values are the means ± SE's for the numbers of individual animals shown in parentheses.

TABLE II. EFFECT OF OVARIECTOMY (OVX) AND TREATMENT WITH 17β-ESTRADIOL (E_2) IN MATURE AND SENESCENT RATS

	Experiments[a]					
	1	2	3	4	5	Mean ± SE[b]
Mature						
Sham	4.8 (3)	8.1 (4)	5.5 (8)	7.9 (4)	—	6.45 ± 0.40 (19)
ovx	6.8 (3)	8.6 (4)	6.3 (3)	7.8 (7)	7.4 (4)	7.52 ± 0.26 (21)
ovx + E_2	—	7.6 (4)	5.3 (3)	7.4 (7)	5.2 (4)	6.78 ± 0.38 (18)
Senescent						
Sham	9.5 (2)	9.5 (3)	9.6 (5)	9.0 (2)	—	9.46 ± 0.31 (12)
ovx	9.3 (3)	—	7.8 (3)	9.8 (5)	7.0 (4)	8.57 ± 0.45 (15)
ovx + E_2	—	—	7.5 (4)	8.4 (7)	8.1 (5)	8.03 ± 0.31 (16)

[a] Values are the mean number of days required for 50% healing for the numbers of animals shown in parentheses.
[b] Values are the means ± SE for the numbers of individual animals shown in parentheses.

healing times for each experimental group. Numbers in parentheses reflect the number of animals per group. Several points can be made from these data.

First, the means for each of the experiments and the total of all individual animals shown in Fig. 2 confirm the finding that senescent rats require about 3 days or 50% longer than young adult animals to reach 50% wound closure (9.46 vs 6.45 days, $P < 0.001$).

Second, ovx increases the time required for young adult rats to reach 50% wound closure by about 1 day ($P < 0.03$). In senescent rats, paradoxically, ovx appeared to decrease mean healing time by 1 day, but this was not statistically significant.

Third, E_2 treatment of young adult, ovx rats restores the rate of healing almost to sham-operated-vehicle levels. Comparison of means within experiments by the paired *t* test, shows that the decrease in healing times following E_2 was statistically significant ($P < 0.05$). Estradiol treatment of senescent rats did not reduce healing time significantly compared with senescent ovx-vehicle animals. However, the E_2-treated group did reach 50% healing about 1.5 days sooner than senescent sham-operated vehicle controls ($P < 0.005$).

Discussion. In the present study full-thickness cutaneous wounds require about 3 days or 50% longer to reach 50% closure in senescent rats than in their younger counterparts. These data are consistent with earlier reports showing that wound healing is delayed in older animals as age increases (13–17) and extends those findings to the senescent end of the lifespan. Age differences in healing rates do not appear to be due to reduced ability to combat wound-related infection, since no differences in wound inflammation or peripheral leukocyte counts were observed between the young adult and senescent groups. Moreover, amoxicillin was included in drinking water to minimize possible age differences in ability to combat infection. After preliminary experiments in the young adults, no attempt was made to separate contraction of the wound from epithelialization although both are likely to be components of the healing process. Wound contraction may have become an even more significant factor if scar formation had been allowed to proceed longer.

The existence of functioning ovaries or estrogen treatment of castrates facilitates wound healing in young adult female rats. The E_2 dose is critical as high dosages appear to decrease healing rates. This finding is in agreement with that of Dyson and Joseph (6), who reported that estrogen dosages of 333 μg/kg body wt inhibited full-thickness wound healing in rabbit ears, while dosages of 33 and 3.3 μg/kg were stimulatory. The dose found to be optimal in this series, 0.33 μg/kg is 10–100 times less; the apparent discrepancy may be due to species differences or to the different type of wound created.

Although ovariectomy decreased the rate

of wound healing in young adult rats, it had no such effect in senescent animals. This is most probably due to the fact that senescent rats exhibit markedly altered ovarian function being mostly anestrus and estrogen deficient (18, 19). The population of Wistar rats used in the present study is 80% anestrus with the rest cycling very irregularly, as described under Materials and Methods. Progesterone levels are also reduced in anestrus rats, and this hormone likewise potentiates wound healing (6, 10). Thus, the removal of nonfunctioning ovaries from aged rats would not be expected to reduce wound healing rates. Nonetheless, estrogen-treated ovariectomized senescent rats healed significantly faster than sham-operated senescent controls. Therefore, when sufficient estrogen is administered, old animals appear to be capable of responding by accelerating wound healing rates. Although the data presented here showing reduced rates of wound healing in aged rates are for females, preliminary studies in our laboratory have revealed a similar reduction in aged male rats. This observation is also consistent with the earlier reports cited above (13–17), many of which are from studies on males. Since androgens as well as estrogens have been shown to potentiate wound healing (6–9), and reductions in both androgen levels and androgen sensitivity have been found in aged rats (20–23) there may also be an endocrine contribution to reduced wound healing in old males.

It is not possible to determine from the present data whether estrogen sensitivity is reduced in the senescent group. The fact that E_2-treated ovx senescent rats do not heal significantly faster than their untreated ovx counterparts is difficult to explain. This observation might suggest decreased estrogenic sensitivity since the comparable young groups show a marked acceleration of healing by estrogens. Certainly, evidence exists to suggest that sensitivity to estrogen may be diminished during aging. Included are reports of decreased induction of acetylcholinesterase in aged rat brain (24) and phosphofructokinase in aged rat uterus (25).

Although the combination of ovariectomy and E_2 administration had a significant effect in improving healing rate in senescent rats, E_2 administration to ovariectomized senescent rats did not have a significant effect, compared with ovariectomy alone. This was because ovariectomy itself yielded a value intermediate between sham-operated controls and E_2-treated ovx animals (but not significantly different from either). Thus the effects of ovariectomy and E_2 treatment seem to be additive. This suggests the possibility that the senescent ovary may produce a substance or substances inimical to healing (e.g., some steroid intermediate, possibly with corticoid-like activity opposed to the action of estrogen).

In any case, age changes in ovarian regulation of wound healing are not sufficient to completely explain the age-related increase in the time required for 50% closure. Even ovx mature rats require only 7.52 days for 50% healing as opposed to 8.02 days for E_2-treated ovx old animals. The difference between these means is not statistically significant, but in all three experiments in which they were compared, the younger group healed more rapidly. It is therefore probable that other endocrine effects as well as neural and intrinsic cellular regulatory processes must be investigated in order to gain more thorough insight into the causes of retarded wound healing during aging. Logical candidates include progesterone and thyroid hormonal control since the former has been reported to accelerate wound healing rates (6) and improve vascularization (10) while the latter increases the rate of cell proliferation and anabolic processes (11).

Finally, cutaneous wound healing, the process of returning damaged skin to physical integrity, includes contraction, cell proliferation, and cell migration (26). Contraction by myofibroblasts, myoepithelial cells and granulation tissue may narrow the wound site while new fibroblasts and epithelial cover replace and remodel the damage (26). The present study has not attempted to examine these aspects of endocrine control of wound healing during aging. Obviously, a next step will be to extend the present findings by detailed analysis of the above processes in order to pinpoint those factors affected by hormones and the aging process.

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