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Type I and Type III Collagen Content of Healing Wounds in Fetal and Adult Rats[1] (42694)

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Abstract. Full-thickness, dermal wounds were surgically created on the dorsa of fetal rats on the 17th day of gestation. The granulation tissue which developed after 2 days (19 days of gestation) was harvested from six to nine animals and pooled and the collagen was extracted with 0.5 *M* acetic acid and acetic acid plus pepsin. The ratio of type III:type I collagen was estimated from densitometer scans of electrophoretically separated α-chains. Full-thickness (to fascia depth) wounds were also produced on the dorsa of adult rats and granulation tissue which had developed for different periods of time up to 30 days was excised. Relative proportions of type III and type I collagen were assessed in normal and granulation tissues taken from the adult rats. Both fetal and adult granulation tissues have elevated type III collagen content but normal fetal tissue has a much higher content of type III than does normal adult tissue. © 1988 Society for Experimental Biology and Medicine.

Dermal wound tissue in the early phases of healing resembles embryonic skin in that type III collagen is present in high proportions relative to type I (1). Granulation tissue which develops in skin wounds is normally resorbed and the scar composition reverts to that of normal tissue (2) but hypertrophic scarring may result if there is some defect in the biosynthesis or degradation of collagen (3). These hypertrophic scars seem to retain embryonic collagen characteristics (4). Normal skin tissue of the fetus has a higher proportion of type III collagen as compared to normal adult skin (5). It has been demonstrated that healing occurs rapidly in A/J mice, with a minimum of scarring, after *intra utero* surgery (6). The reduction of scarring in these tissues may be related to the type of collagen which is normally present and that laid down in wounds, or it may represent a good balance between collagen synthesis and degradation. It was of interest to see if fetal wounds which were created *in utero* had type III collagen elevated above that of normal fetal skin. The results reported here compare the type III/type I collagen ratio in normal skin and granulation tissues from dermal wounds in adult and fetal rats.

Materials and Methods. *Production of wounds on fetal rats.* After 17 days of gestation Sprague–Dawley rats were anesthetized by im administration of sodium pentobarbital, ketamine, glycopyrrolate, and chloropromazine. Linear abdominal incisions were made and one horn of the uterus was digitally palpated to bring a fetus into the proper orientation. Hysterotomies (linear, 3–4 mm) were performed to expose only a small portion of the fetus to be wounded. Wounds were produced under a Zeiss operating microscope using scalpel and scissors to remove a 5- to 15-mm^2 full thickness of skin to fascia depth (See Fig. 1A). Except for a few facial wounds the incisions were made on the dorsa of the animals. Two to four fetuses per mother (four to six gravid animals per experiment) were wounded and the uterus and abdomen of the mother were sutured. At 19 days of gestation (full term = 21 days) wounded fetuses were removed (see Fig. 1B), granulation tissue was excised, pooled, and extracted as described below. Normal fetal skin was obtained from the dorsa of unwounded animals randomly selected from most of the mothers in an experiment. Skins from unwounded fetuses were pooled and the collagen was extraced as described below.

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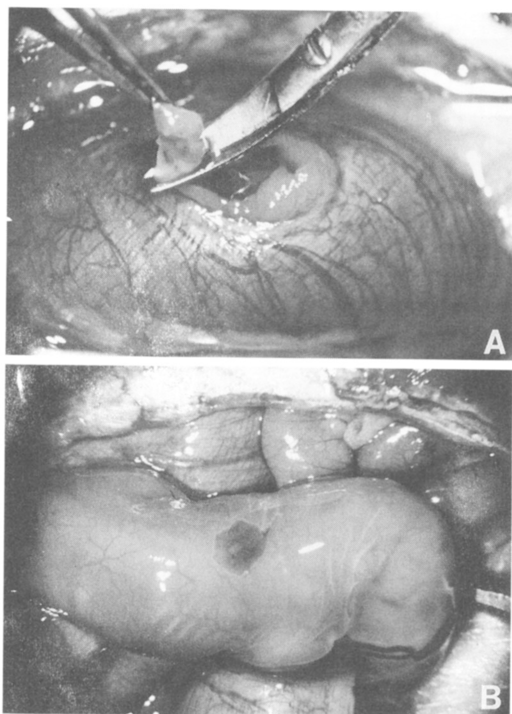

FIG. 1. Creation and harvesting of fetal wound tissue. (A) Hysterotomy performed at 17 days of gestation under a Zeiss, Model OPMI6SPH operating microscope using scalpel and scissors. A 5- to 15-mm^2 wound was created at full epidermal/dermal depth to the supporting fascia. (The fetal tissue is being removed in this photograph.) (B) Granulation tissue in a fetal wound at the time of tissue harvesting (19 days of gestation; 2 day *in utero* scar).

Production of wounds on adult rats. Adult (400–450 g) male Sprague–Dawley rats were depilated on their dorsa, anesthetized as described above, and two, 1 × 2-cm full thicknesses of skin surgically removed to the depth of fascia. At various times (2–30 days) after wounding, the animals were anesthetized and the granulation tissue carefully excised from the healing wounds and used for collagen analysis. Animals were sacrificed after the last scar tissue was removed.

Collagen extraction. Surgically excised rat skin samples were immediately immersed for approximately 30 min in 10 ml of ice cold 0.05 *M* tris(hydroxymethyl)aminomethane (Tris)–HCl, pH 7.5, which contained 4.5 *M* NaCl, 0.02 *M* disodium ethylenediaminetetraacetate (EDTA), 1 m*M* phenylmethylsulfonyl fluoride (PMSF), and 2 m*M* *N*-ethylmaleimide (NEM). Samples were then rinsed briefly in cold distilled water, blotted, and quickly weighed. Collagen was extracted from these tissues by a modification of the procedure of Miller and Rhodes (7).

Portions of the tissue were homogenized in 10–20 vol of ice cold 0.5 *M* acetic acid (adjusted to pH 2.5) using a Tissue Tearor (Biospec Products). Extraction was continued for 4 days at 4°C on a New Brunswick or Burrell shaker. The samples were then sedimented at 30,000*g* for 45 min in a refrigerated centrifuge and the supernatant fluids saved.

The tissue pellets were resuspended in the original volume of acetic acid, and pepsin (Sigma) was added (20 mg/g wet tissue). Pepsin digestion proceeded overnight with shaking at 4°C, insoluble material was removed by centrifugation (30,000*g*, 1 hr) and the supernatant fluid was pooled with the first acetic acid extract. The pooled extracts were dialyzed in 7 liters of 0.02 *M* Na_2HPO_4 (pH 8.2) overnight at 4°C. The precipitated collagens were collected by centrifugation, redissolved in a minimum volume of 0.5 *M* acetic acid, dialyzed against 4 liters of the same solution, and lyophilized.

Total collagen determination. Collagen content of tissues was estimated on the basis of hydroxyproline content assuming that collagen contains 12.5% hydroxyproline (8).

Determination of type I and III collagens. Lyophilized collagen samples were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) in the presence of 3.6 *M* urea (9). Samples, at a concentration of 2.5 mg/ml, were dissolved in SDS binding buffer (10), boiled for 3 min, cooled, and 5 to 10 μl were loaded into wells of 5% polyacrylamide gels. Rat tail tendon collagen was used as a type I standard and purified types III and V standards were purchased from Sigma Chemical Co.

Electrophoresis was conducted for 3 hr at constant current (30 mA per slab). The gels were stained overnight in Coomassie blue R-250, and destained for 1 hr in 50% methanol, 10% acetic acid followed by complete destaining in 7% acetic acid–5% methanol. After destaining the gels were scanned with a Hoefer CS-300 densitometer operating in the transmittance mode. Areas of the $\alpha1$(I) and $\alpha1$(III) peaks were calculated manually and

the percentage of type III collagen was determined from the formula of Chan and Cole (11):

$$\frac{\text{Area } \alpha 1(\text{III}) \times 1.12 \times 100}{[\text{Area } \alpha 1(\text{III}) \times 1.12] + \text{Area } \alpha 1(\text{I})}.$$

Results. With the exception of one experiment (not included in the data), all of the collagen determinations were done on dorsal tissues. Dorsal wound sites were randomly scattered, but it was noted that midline or cranial wounds developed a thin wound covering which was more difficult to remove than those which were lateral to the midline (Fig. 1B).

A survival rate of approximately 70% was experienced among the fetuses which received wounds. Several wounded fetuses were allowed to go to full term after their viability was verified at 19 days of gestation. Although the granulation tissues were not examined, it was obvious that healing had progressed appreciably during the 19th to 21st day of gestation (data not shown). Table I shows the percentage of type III collagen relative to type I in the skins of normal (unwounded) rat fetuses and the granulation tissue from surgically created fetal wounds 2 days after wounding. In each of the six different experiments the entire, full-thickness granulation tissues from all of the wounded fetuses were pooled to provide a sufficient amount for collagen analysis. Normal skins of one or two unwounded fetuses taken from most of the mothers on which surgery was performed were also pooled to randomize the samples. The number of mothers which provided fetuses for both normal and granulation tissues is also shown in Table I.

The percentage of type III collagen in the granulation tissue taken at different times from dorsal wounds which were surgically produced in adult male rats is presented in Table II. Each rat used in the experiments was wounded at two dorsal sites and granulation tissue was taken from a single animal at two different times (2–30 days postwounding). Full-thickness skin patches which were removed to create wounds on six different animals were used as normal tissue. Table III compares the average value of fetal normal and granulation tissues (Table I) with that of corresponding tissues taken from adult rats (Table II). The age of the granulation tissue (healing period) was 2 days in both fetuses and adult.

The efficiency of our collagen extraction procedure was determined by hydroxyproline analysis of the residue remaining after acetic acid/pepsin extraction of adult skin

TABLE I. TYPE III COLLAGEN CONTENT OF EXTRACTED NORMAL AND WOUND TISSUE FROM THE DORSAL SKIN OF FETAL RATS

	Normal fetal tissue[a]		Fetal granulation tissue[b]		
Experiment	No. of fetuses	% Type III collagen	No. of fetuses	% Type III collagen	Relative type III collagen content wound/normal
1	4 (3)[c]	23.5	7 (5)[c]	35.7	1.52
2	8 (4)	34.0	6 (4)	35.0	1.03
3	8 (5)	22.6[d]	8 (6)	28.4	1.32
4	4 (2)	29.1	6 (4)	27.6	0.95
5	5 (5)	24.4	9 (6)	39.7	1.63
6	5 (5)	22.8	9 (5)	34.7	1.52

[a] A segment (approx 0.5 × 1.0 cm) of skin to fascia depth was obtained from the dorsum of one or, at most, two unwounded fetuses taken from a mother which had also provided the granulation tissue from one to three wounded fetuses. The normal tissues, collected in each experiment at the time granulation tissue was harvested, were pooled and the collagen extracted as described under Materials and Methods.

[b] Granulation tissue from all of the surviving, wounded fetuses in an experiment was collected 2 days after wounding and pooled and collagen was extracted as described under Materials and Methods.

[c] Number of mothers providing the given number of fetuses.

[d] An average of five separate determinations on the same pool of unwounded (normal) fetal skins. Average deviation ± 1.7; range 19.6 to 26.6%.

TABLE II. TYPE III COLLAGEN CONTENT OF EXTRACTED NORMAL AND WOUND TISSUE FROM THE DORSAL SKIN OF ADULT RATS

Days after wounding	% Type III	Relative type III collagen content wound/normal
0 (normal)	14.9[a]	
2	30.6	2.04
4	26.4	1.76
6	27.0	1.80
8	28.6	1.91
10	29.3	1.95
12	32.1	2.14
17	35.9	2.39
20	27.9	1.86
26	24.1	1.61
30	26.3	1.75

[a] Average of six experiments; average deviation = ±0.98; range 13.2 to 16.4%. All other values are from single determinations.

and 2-day-old granulation tissue samples. Collagen extraction was virtually complete (99.6%) from the granulation tissue and slightly lower from normal adult skin (89.9%).

Samples (10–21 mg) of fetal and adult normal skin and wound tissues were lyophilized and the total collagen content measured by a hydroxyproline assay (8). The total amount of collagen varied considerably with the age of the animals and was low in fetal and adult granulation tissues compared to comparable unwounded tissues (Table IV).

Discussion. Several different collagen extraction procedures were attempted before we adopted the procedure used in these studies. We noted in early trials that when the 0.5 *M* acetic acid extract was not pooled with the pepsin extract, a much smaller amount of type III collagen was recorded in fetal and newborn rat skins. Consequently, we retained all of the solubilized collagens which were produced by soaking the tissues in 0.5 *M* acetic acid for 4 days followed by digesting for 18 hr with pepsin in acetic acid solution. This differs from the Chan and Cole (11) procedure because they deliberately discarded the 24 hr (at 4°C) acetic acid extract of their human tissues. Our procedure gave us reasonably consistent values for the relative amounts of type I and III collagens in both normal and granulation skin tissues from animals at widely different developmental stages. As recommended by Sykes *et al.* (12), a greater proportion of collagen was brought into solution for quantitation.

TABLE III. COMPARISON OF NORMAL AND GRANULATION DERMAL TISSUE TAKEN FROM ADULT AND FETAL RATS

	Average % type III[a]	
	Normal skin	Granulation tissue 2 days after wounding
Fetal	26.5 ± 3.6	33.3 ± 3.7
Adult	15.0 ± 1.0	30.6[b]

[a] Averages of values shown in Tables I and II.
[b] A single determination.

TABLE IV. TOTAL COLLAGEN CONTENT OF TISSUE SAMPLES AS DETERMINED BY HYDROXYPROLINE ANALYSIS

	Collagen content	
Skin samples	μg/mg dry wt. (no. rats)	Range
Adult, normal	331 (4)	230–464
Adult, 2 day scar	50 (2)	49–52
Fetal, normal	17 (2)	14–21
Fetal, 2 day scar	6 (1)	—

Note. Hydroxyproline assayed and collagen content calculated as described under Materials and Methods.

Table IV illustrates the variability of collagen content in the various rat skin samples. Total collagen per milligram of tissue was lower in fetal tissues than in adult tissues. Wound tissue from both fetal and adult skins had lower contents of collagen than normal skins and was lowest in fetal wound tissue. This added to the difficulty of extracting workable yields of collagen from samples and also may have contributed to the greater range of results.

Our results are consistent with those of other investigators who found a higher percentage of type III collagen in fetal skin compared to adult skins (compare normal values in Tables I and II) (5). We also show that in fetal granulation tissue there is a slight increase (average of 1.3 times) in the percent-

age of type III collagen over that in normal fetal skin (Table I). This agrees with what others have reported in both juvenile and adult skin wounds (e.g., (13, 4, 14)).

There was a slight upward trend in type III collagen between 4 and 17 days (Table II) but we did not see a reversion to normal values in adult scars up to 30 days old. It should be noted that we analyzed only the remaining central granulation tissue of all wounds.

Our main objective in these studies was to see if there was any correlation between the apparent accelerated healing capabilities and collagen metabolism of the fetus compared to those of the adult rat. Both fetal and adult granulation tissues are higher in type III collagen compared to normal skins (Table III). However, fetal granulation tissue has a type III composition which is only slightly elevated above that of normal fetal skin, whereas adult, 2-day-old granulation tissue has a type III composition which is approximately twice that of normal adult skin. We have observed that fetal wounds, which are closer in composition to surrounding tissue, heal readily and with a minimum of scarring compared with normal adult skin. Collagen that accumulates in adult skin during the repair process differs considerably from the original (normal) tissue (Table III). Presumably events that occur during tissue repair are similar to those of normal development processes. Fibroblasts active in collagen synthesis in healing wounds are more active than cells from normal skin and may have reverted to an embryonic phenotype (15). In the case of the fetuses, their skin composition is already "embryonic-like," actively undergoing differentiation and development, and in a log phase of division and synthesis, hence, healing is accelerated.

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