

Full Length Research Paper

The content and ratio of type I and III collagen in skin differ with age and injury

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Accepted 4 February, 2011

The aim of this study is to examine type I and III collagen content and distribution in skin within the contexts of patient age and injury, as well as to elucidate possible mechanisms of hypertrophic scar formation. Normal human skin and hypertrophic scar specimens were obtained from spontaneously aborted fetuses and burn patients of different ages (adolescent group, ≤ 18 years; adult group, >19 and ≤ 50 years; elderly group, >50 years). Total collagen content was indirectly determined by quantification of hydroxyproline. Levels of type I and III collagen as well as the ratio of type I/III were determined by immunohistochemistry and image analysis. Results obtained showed that the mean content of type I and III and type I/III ratio in normal skin differed significantly among age groups ($P < 0.05$), with the lowest levels of type I, III, and the highest ratio of type I/III observed in the elderly age group. Differences between normal uninjured skin and hypertrophic scar tissue were significant for all investigated parameters within any of the three age groups examined (adolescent, adult, elderly; $P < 0.05$). Content of type I, III collagen and type I/III ratio also exhibited age-dependent differences during recovery in hypertrophic scar specimens. Thus, type III collagen synthesis decreases with age resulting in a skewed type I/III ratio and changes in skin tension, elasticity, and healing. Also, the content of type I, III collagen and type I/III ratio are significantly altered in hypertrophic scar tissue compared to uninjured age-matched controls, resulting in a different structural organization that is also influenced by patient age.

Key words: Age group, hypertrophic scar, collagen type I, collagen type III, immunohistochemistry, hydroxyproline.

INTRODUCTION

Collagen is the most abundant protein produced by mammals, and it is fundamental in contiguous formation of the interstitium through the epidermis. Type I and III collagen are formed in human skin in a higher proportion relative to other types, and are maintained in a fixed proportion relative to one another in normal skin tissue. However, in human formation of scar tissue, as a result of age or injury, there is alteration in the abundance of type I and III collagen as well as their proportion to one another. Recently, both the abundance and balance of type I and

III collagen have received considerable research attention (Feng et al., 2001; Garner et al., 1993; Ghahary et al., 1996; Guan et al., 1997; Guo., 2002; Hurley et al., 1993; Ichiki et al., 1997; Kennedy et al., 1995; Linares, 1996; Liu et al., 2001; Lu, 2003; Shah et al., 1995; Tan et al., 1993; Tang et al., 2004; Thomas et al., 1995; Wan et al., 2001; Wang et al., 1999; Wu et al., 2000; Yin, 1999; Zhou et al., 1997). In the present study we aim to identify the principles and causes surrounding age-dependent variations in type I and III collagen levels and ratio by examining skin tissue from a span of age groups, and comparing inconsistencies in collagen content and proportion among normal and hypertrophic scar skin specimens.

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Abbreviations: BSA, Bovine serum albumin; PBS, phosphate buffered saline; DAB, 3,3'-diaminobenzidine; SABC, streptavidin-biotin-peroxidase complex.

MATERIALS AND METHODS

Tissue samples

Normal skin samples were provided by Beijing jishuitan hospital,

and were procured from spontaneously aborted fetuses (n=10) and patients treated for burns that required skin grafts (n=30; of which male =16 and female =14). Skin isolated for grafts consisted of whole thick skin from the thigh or mid-section. As physiological characteristics of skin change at a predictable rate in relation to age, tissue samples were classified into the following groups based upon patient age: Fetus group, adolescent group (\leq age of 18 y), adult group (>19 y, ≤ 50 y), elderly group (>50 y). A total of 10 samples per experimental group were analyzed. Samples of post-burn hypertrophic scar tissue (n=90; male = 53 and female =37) were sampled during the course of recovery (6-12 months, 12-24 months, >24 months post burn) and classified by age as described above (adolescent group, adult group, elderly group; n=10 samples per group). All patients and their families were aware and agreed to tissue donations, and they also confirmed that hormonal or topical drugs were not used by the patient during the last 12 months.

Immunohistochemistry

Tissue samples were fixed in 4% formaldehyde for 12 h, dehydrated, and olefin-embedded prior to sectioning at 3 μ m. Type I and III collagen were assessed in fixed sections using polyclonal antibodies and the streptavidin–biotin–peroxidase complex (SABC) kit. Briefly, slides were confined with 50 g/L bovine serum albumin (BSA), and incubated with 50 μ l of primary antibody (mouse anti-human Type I collagen or mouse anti-human Type III collagen) diluted 1:100 in 0.01 mol/L phosphate buffered saline (PBS), pH 7.3 for 1 h at room temperature followed by incubation overnight at 4°C. The following day slides were acclimated to room temperature for 1 h prior to incubation with 50 μ l of biotinylated anti-mouse IgG for 1 h at room temperature, after which they were incubated with SABC solution for 1 h at room temperature followed by 10 min in 3,3'-diaminobenzidine (DAB). Slides were washed in PBS in-between incubators. A positive reaction was observed as brown staining. The area of positive immunostaining was quantified using a computer image analysis system to calculate the ratio of positive to negative staining. Data is expressed as the mean of three randomly selected fields of view.

Quantification of hydroxyproline

Fresh specimens (0.3 g) that were stored in isotonic saline at 4°C were dissected and homogenized on wet ice. Collagen content was estimated by the quantification of hydroxyproline using the hydroxyproline kit (Nanjing, China) according to the manufacturer's instructions. Briefly, the homogenate was centrifuged for 10 min at 986 x g. The absorbance of the supernatant containing hydroxyproline was measured by spectrophotometry at a wave length of 550 nm. The density of hydroxyproline was calculated using the following equation:

$$\text{Hydroxyproline } (\mu\text{g/ml}) = \frac{\text{Absorbance value tested} - \text{absorbance value untested}}{\text{Standard absorbance value} - \text{absorbance value untested}} \times \text{Standard test tube density } (\mu\text{g/ml})$$

Specimen hydroxyproline content was calculated using the following equation:

$$\text{Hydroxyproline } (\mu\text{g/g}) = \frac{\text{Density of hydroxyproline} \times \text{volume of liquid supernatant (ml)}}{\text{Mass of specimen}}$$

Gross collagen protein content of a specimen was then estimated using the equation (Medugorac I., 1982):

$$\text{Gross collagen content} = \text{Hydroxyproline content} \div 13.4\%$$

Statistical analysis

Data is expressed as mean (x) \pm standard deviation (s). Variance and differences among means were determined by analysis of variance (ANOVA) test using SASV 8 Stat Software. Statistical significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Type I and III collagen immunostaining

Positive immunostaining for Type I collagen was thick and heavy, with a widely distributed staining pattern (Figure 1). Conversely, type III collagen immunostaining was light and sparse with an uneven distribution (Figure 2). Signal among the interstitial cells of the dermal layer in hypertrophic scar tissue was robust and extensive for type I collagen (Figure 3) and weaker although clearly present for type III collagen (Figure 4).

Type I, III collagen content

As shown in Table 1, type I, III collagen content varied significantly among the four age groups ($P < 0.05$). The ratio of type I to type III collagen increased with patient age, with a significant difference observed in the youngest (fetus) and the oldest (elderly) groups ($P < 0.05$). Type I and III collagen content, as well as the ratio of type I to III, in hypertrophic scar tissue during the course of recovery among different age groups is summarized in Table 2. Means differed significantly between normal uninjured skin and scar tissue specimens within a given age group, among age groups, as well as during recovery for all indices examined.

In healthy human skin, type I and III collagen have relatively substantial roles during collagen formation, comprising of 80-85% and 10-15% in human skin, respectively (Riita et al., 2002). Given the importance of collagen types during skin formation and their presumed fluctuations in content and relative ratio during aging, we questioned the magnitude of change associated with age and injury. Visualization of protein distribution patterns of the two types of collagen revealed that type I collagen was a major contributor in the formation of skin, conclusions were based on skin thickness, while type III collagen had greater involvement in establishing the reticular structure of skin as protein expression was punctuated and diffuse. In terms of morphology, a negative correlation was observed between fiber diameter and the ratio of type I/III collagen content. We also observed differences between scar tissue and uninjured normal skin, specifically in collagen content and ratio of type I/III. Morphological examination supported the differences observed as type I collagen immunopositive fibers were thick, forming the foundation of scar tissue during fibrosis. Morphological differences between scarred and normal skin are likely due to the progressive accumulation of

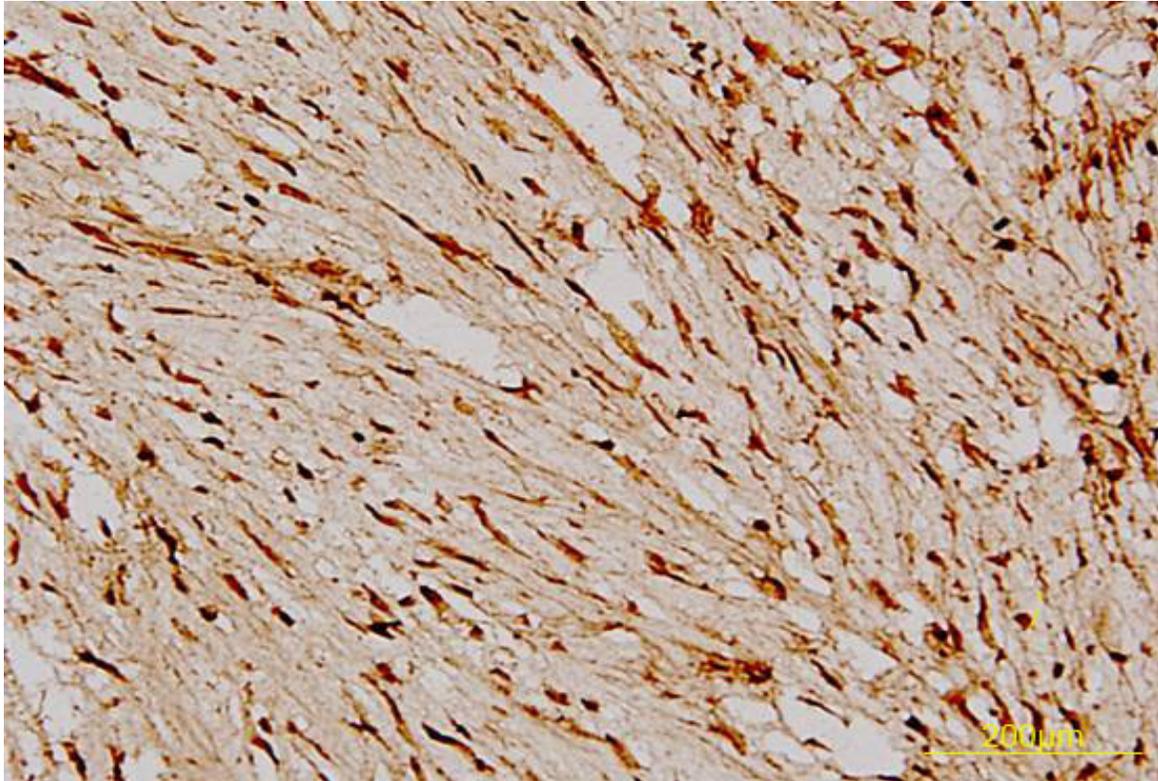


Figure 1. Type I collagen was assessed in normal skin tissue by immunohistochemistry. Brown staining represents positive immunostaining, and was observed within the dermal layer.

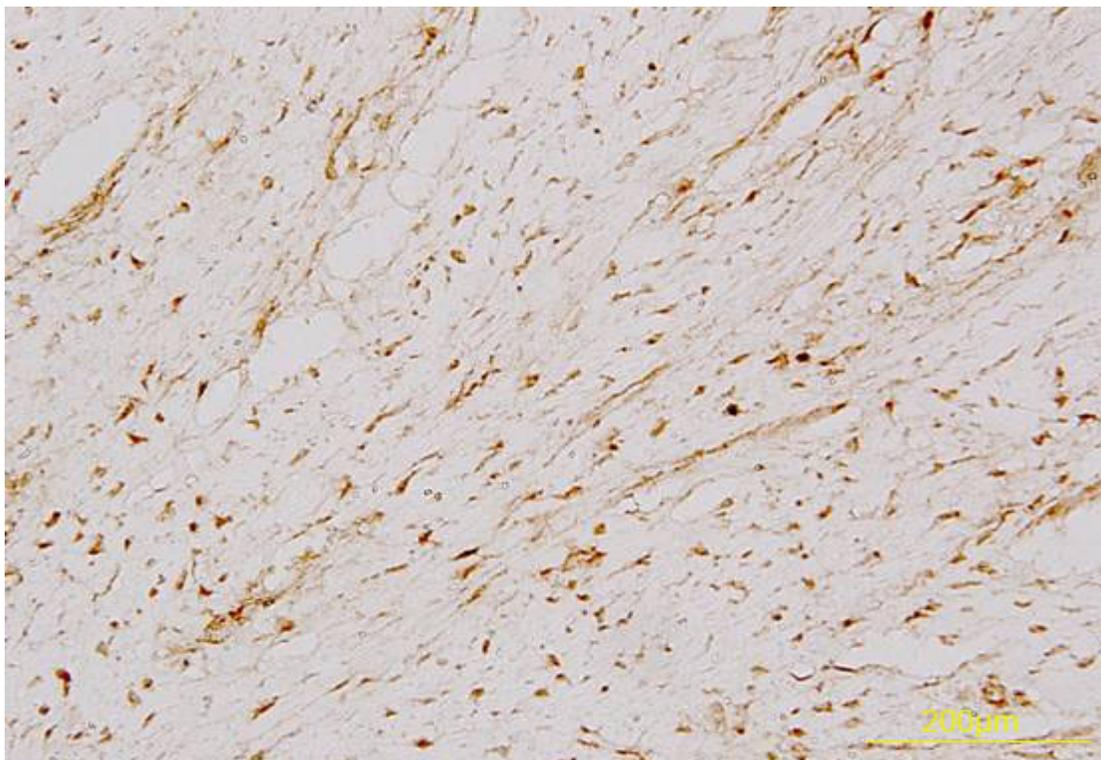


Figure 2. Type III collagen was assessed in normal skin tissue by immunohistochemistry. Light brown staining represents positive immunostaining, which had an uneven distribution within the dermal layer.

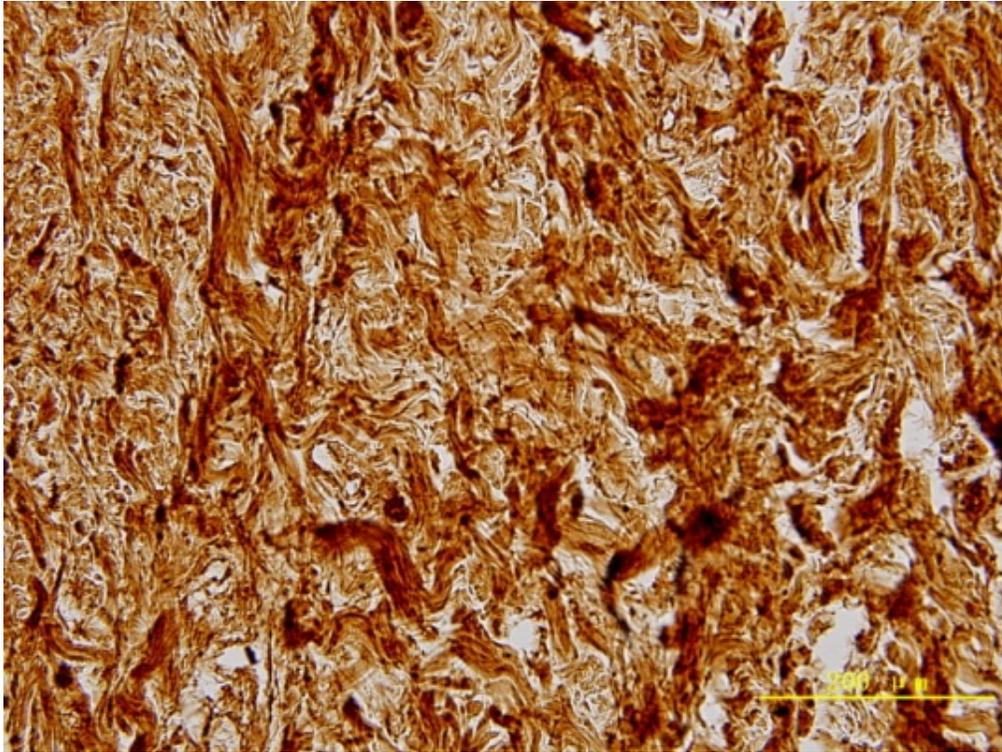


Figure 3. Type I collagen was assessed in skin hypertrophic scar tissue by immunohistochemistry. Positive immunostaining (brown staining) was robust and extensive within the dermal layer.



Figure 4. Type III collagen was assessed in skin hypertrophic scar tissue by immunohistochemistry. Positive immunostaining (brown staining) was light and unevenly distributed in small collagen bundles within the dermal layer.

Table 1. Type I, III collagen content in normal skin (mean \pm s).

Age group	Specimens (n)	Type I collagen ($\mu\text{g/g}$)	Type III collagen ($\mu\text{g/g}$)	Type I/III
Fetus	10	264.71 \pm 5.88 ^a	278.87 \pm 6.18 ^a	0.95 \pm 0.03 ^a
Adolescent	10	279.12 \pm 7.65 ^b	123.27 \pm 5.30 ^b	2.27 \pm 0.13 ^b
Adult	10	241.79 \pm 8.23 ^c	98.41 \pm 5.58 ^c	2.46 \pm 0.15 ^c
Elderly	10	209.50 \pm 14.31 ^d	71.30 \pm 7.41 ^d	2.97 \pm 0.40 ^d

Different letters represent statistically significant differences in mean across age groups ($P < 0.05$).

Table 2. Type I, III collagen content ($\mu\text{g/g}$) in skin hypertrophic scar tissue (mean \pm s).

Age group	Specimens (n)	Type of collagen	Normal specimen	Course of recovery (months)		
				6 -12	12-24	> 24
Adolescent	30	I	279.12 \pm 7.65	523.12 \pm 17.76	541.75 \pm 17.13	517.65 \pm 12.79
		III	123.27 \pm 5.30	89.62 \pm 4.73	104.68 \pm 7.20	91.79 \pm 7.34
		I/III	2.27 \pm 0.13	5.85 \pm 0.40	5.19 \pm 0.30	5.66 \pm 7.34
Adult	30	I	241.79 \pm 8.23	469.09 \pm 13.87	485.76 \pm 8.20	450.63 \pm 12.05
		III	98.41 \pm 5.58	124.56 \pm 12.67	130.96 \pm 9.99	112.87 \pm 4.58
		I/III	2.46 \pm 0.15	3.80 \pm 0.36	3.73 \pm 0.32	4.00 \pm 0.13
Elderly	30	I	209.50 \pm 14.31	403.66 \pm 5.78	411.88 \pm 5.40	379.71 \pm 9.37
		III	71.30 \pm 7.41	151.75 \pm 10.06	159.89 \pm 5.86	131.78 \pm 6.40
		I/III	2.97 \pm 0.40	2.67 \pm 0.22	2.58 \pm 0.09	2.89 \pm 0.19

Different letters represent statistically significant differences in mean across and within age groups ($P < 0.05$).

type I collagen. Although the scarred skin was stiff, rough, dry and inflexible compared to uninjured skin, the scar matured over time; as the content of type I collagen increased, type III collagen declined (liu et al.,2001).

We also observed age-dependent differences, as total collagen content in normal skin declined with age, particularly type III collagen which translates to a progressive increase in type I/III ratio, a factor that may be associated with the prevalence of permanent scar tissue. One such factor present in the fetus that may prevent scar tissue formation is sufficient content of type III collagen. Speculation of a low ratio of type I/III collagen content could mean greater flexibility, and in the presence of an injury, a greater tensility during recovery translate to a well defined tissue reformation, akin to uninjured tissue. Type I and III collagen will function in this context during wound recovery. Type III collagen is the first to emerge and acts as a bridge to the wound, after which type I collagen appears in conjunction with type III during tissue reformation to build a solid bracket and facilitate wound healing while circumventing the formation of scar tissue. While the ratio of type I/III collagen content is above 1 for adolescent, adult, and elderly age groups, type I and III collagen content are lower in the elderly age group and skin elasticity and tensility were reduced relative to other age groups. The age-dependent increase in the ratio of type I/III content observed, is consistent with previously reported findings (Qiu et al.,2003; Rong et al., 2008). When we examined type I and III collagen content in

hypertrophic scar tissue, gross collagen content, type I in particular, was significantly greater compared to uninjured skin. Among all three age groups investigated, injured skin differed significantly during recovery from age-matched uninjured controls for type I and III content and type I/III ratio. Differences among age groups in hypertrophic scar tissue specimens were also observed, as both type I collagen content and the ratio of type I/III collagen during recovery were inversely proportional to age, while type III content increased with age. It is very important to note that in the youngest age group examined (adolescent), which has the highest type I collagen content and type I/III ratio and the lowest type III content, immature fiber cells and an instable interior environment may have sensitized fibroblasts to secrete type I collagen or any number of growth factors. Therefore, excessive secretion of type I collagen may result in disorganized fiber structure and hypertrophic scar formation. Resultant data in adult and elderly age groups are in concert with the presence of fully mature fibroblasts, as type I and III collagen content decreased and increased, respectively. Recovery progresses yielded a stable type I/III ratio. A stable type I/III ratio would limit sensitivity to growth factors and any consequential secretion of collagen fiber thereby lessening the possibility of hypertrophic scar formation in comparison to the adolescent age group. Age-dependent differences in the levels of investigated variables were also observed during recovery. We hypothesize that type I collagen

content was the basis of hypertrophic scar formation, with differences in the content of type I collagen translating to a difference in variables and morphology between hypertrophic scar and uninjured skin specimens.

Collagen is a keystone of skin formation and repair, playing a crucial role in the maintenance of skin tensility and elasticity. Variations in content and ratio provide the basis of hypertrophic scar formation; thus, data presented here on age-dependent levels of collagen types in normal and recovering skin may provide a foundation for novel clinical treatment of scarring and prevention of hypertrophic scar formation.

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