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# Temporal and Spatial Distribution of Collagen Types I and III during Experimental Wound Healing An Immunohistochemical Study

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**Introduction**

Collagen is a structural protein of connective tissues (cartilage, tendons, ligaments) and organs (skin, heart, liver, kidney, lungs, blood vessels, bones). Collagen is a family of fibrous protein that is a component of the extracellular matrix (ECM). It is made up of three alpha chains that wrap around each other to form the collagen fibers. The overall architecture of the collagen and its type depend on the amino acid sequence of the chain. Collagen can be divided into interstitial collagen, basement membrane collagen, and peripheral collagen according to its location in the body. There are over 30 types of collagen identified. Three left hand helices (proline II), wound together and linked to each other to form a long, strong right hand helix, is the normal collagen molecule, the triple helix. In distensible connective tissues such as skin, lung, and vasculature, collagen type I (Col I) is typically found together with type III collagen (Col III). The major collagens in ECM are collagen type I and III, but collagen type IV, V, VI, and VIII are also found in ECM. Collagen type I forms a scaffold with thick fibers and low turnover. Collagen type I maturation, however, is dependent on collagen type III, which forms thin, weak fibers with rapid turnover. Also, collagen types I, II, and III are the most abundant fibrillar collagens. Collagen type I is found in skin, tendons, blood vessels, lungs, heart, and other organs, and it is the major organic component of the calcified tissue of bones and teeth. But reticular fibers are collagen type III which are usually found with collagen type I( Khalaf et al.,2019).

Collagen type I and collagen type III, which provide structural support to the muscle cells and are important for cardiac function, are the major structural proteins in the ECM of the myocardium. Myocardial collagen protein levels are altered in DCM,

which is characterized by Col I deposition. Because of their different mechanical properties, the ratio of collagen types in the heart matters. Prior study used electron microscopy and immunohistochemistry to demonstrate increased collagen fibers in end-stage DCM. Another biochemical study showed an increase in the absolute amounts of Col I and Col III. But some studies found opposite results where a marked increase in thin collagen fibers and a decrease in thick collagen fibers were observed in the heart. In a recent study, patients with myocardial infarction and coronary artery bypass graft had significantly lower collagen type III in the aortic wall samples than patients with stable angina. Also, collagen type III is more susceptible to alterations in the local vascular wall resulting in unstable atherosclerotic plaque because it is thin, less stable, and more prone to inflammatory responses. The elevated collagen gene expression is controlled by the Col I and Col III gene promotors with TGF-β-related regulatory elements. Because the Col I promotor also has several SP1 binding sites that the Col III promotor does not, the different gene expression levels of Col I and Col III may depend on their promotors. The difference in the Col I and Col III levels may be due not only to differential gene expression but also to differential degradation of both proteins. Two MMPs are reported to be active in the degradation of collagens. MMP1 is produced by fibroblasts and has equal affinity for Col I and Col III degradation. MMP-8, on the other hand, produced by neutrophils, has a greater affinity for Col III. Thus, differential activities or expression levels of MMP may also contribute to alterations in myocardial Col I and Col III content, besides the gene expression (Kamar et al.,2019).

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Type III collagen (COL3), the second most abundant collagen in the body, is essential for fetal development and postnatal tissue maintenance and repair. While a regulatory function for COL3 in skin wound healing has been suggested for decades based on its early induction during healing, our lab first demonstrated a defined role for COL3 in controlling skin repair and matrix remodeling/scarring by limiting myofibroblast activation and persistence. Moreover, elevated COL3 is found in two mammalian models of scar-free skin wound healing, the midgestational fetus and the African Spiny Mouse (Acomys spp), but the spatiotemporal effects and mechanisms by which COL3 promotes regenerative responses are poorly understood in these models and in other tissues. Here, we explore the mechanistic functions of COL3 in driving fibrillar collagen network formation that supports effective re-epithelialization and inhibits scar formation. Since COL3 controls matrix architecture, we investigated collagen architecture in regenerative (Acomys cahirinus) and scar-permissive (Mus, Col3B6/B6, and Col3F/F) healing microenvironments and employed second harmonic generation (SHG) microscopy to identify critical collagen microarchitectural features that drive a transition between scarless and scarring wounds. Moreover, we show that COL3 enhances cutaneous wound healing efficacy and quality by accelerating re-epithelialization and restricting scar-promoting fibroblast activation and collagen deposition. Our data also indicate that COL3 controls fibroblast mechanoperception independent of elevated GT stiffness in an α11 integrin–dependent manner, offering novel insight into how COL3 may orchestrate dynamic reciprocity between stromal cells and collagens in repair and regeneration of cutaneous and noncutaneous tissues and the tumor

microenvironment. Characterizing such mechanisms has implications for refining COL3-targeted therapies that can be exploited to better treat diseases of the wound healing–fibrosis–cancer triad( Kisling rt al.,2019).

Tissue repair is a highly coordinated process involving multiple cell types, soluble factors, and ECM proteins and their modifiers, which is marked by dynamic reciprocity, where cells and the ECM interact and respond to each other over time. Importantly, changes in collagen structure, density, type, and tissue stiffness offer topographical, biochemical, and biomechanical cues to cells following injury. Collagens play a double role in skin wound healing. While collagen deposition is necessary for effective re-epithelialization and dermal reconstruction in cutaneous wound healing, the substitution of normal skin structures by collagen leads to scar tissue, which is functionally inferior to uninjured skin. In addition, over deposition of collagen leads to pathologic scar formation. Scars are generally described as having an anisotropic alignment of collagen fibers parallel to the neoepidermis, as opposed to the unwounded dermis where collagen fibers are bi-isotropically aligned in a basket-weave pattern. But the features of a proregenerative collagen matrix that can recapitulate the structure and function of intact skin are poorly understood (Połomska et al.,2021).

## ❖ Collagen Metabolism

The collagen network is a dynamic structure with collagen turnover, probably between 80 and 120 days, depending on the balance between collagen synthesis and degradation. The alteration of collagen number relies on fibroblasts, especially the fibroblasts that have differentiated into myofibroblasts, the phenotype responsible for collagen turnover. These cells respond to mechanical strain, locally produced autocrine and paracrine factors (e.g., TGF-β, growth factors such as angiotensin II), and hormones (e.g., aldosterone) delivered from the circulation. Fibroblast and myofibroblast activity is also regulated by several proinflammatory cytokines secreted by monocytes and macrophages. The capacity of these cells to synthesize and secrete fibrillar collagen precursors (the two more abundant procollagen types in the heart, Col I and III) and enzymes that process procollagen precursors to mature fibril- and fiber-forming collagen (procollagen proteinases and lysyl oxidase) is dependent on alterations in their rates of proliferation and migration and alterations in response to all of the above. Many clinical studies of candidate biomarkers of collagen metabolism are divided into (i) biomarkers of collagen molecule synthesis that build new collagen fibers and (ii) biomarkers of collagen molecule degradation that incorporate the old fibers (Thankam et al.,2019).

- Collagen synthesis

Fibroblasts (resident and myeloid cell trans-formed fibroblasts) are the major source of newly synthesized collagen in the healing wound. Biosynthetic events of fibril-forming collagens are the most studied of all collagens, requiring temporal and spatial coordination of multiple biochemical events. In the ER, the pre-pro-collagen is processed to pro-collagen by cleavage of the signal peptide at the N-terminus following transcription. The formation of the triple-helical structure characteristic of collagens is due to the hydroxylation and glycosylation of amino acid residues The pro-collagen triple-helix is stabilized in the Golgi for further processing and maturation and packaged into secretory vesicles that are released into the extracellular space where the pro-collagen is enzymatically cleaved into tropocollagen. Covalent cross-linking builds the final collagen fibril. This cross-linking is responsible for the mechanical properties (elasticity and reversible deformation) of fibrillar collagens. These crosslinks include cystine-cystine disulfide bonds, transglutaminase cross-links, advanced glycation end (AGE) product cross-links and reducible and mature lysyl oxidase pathway cross-links. Cross-linking degradation is dependent on collagen type and tissue environment, leading to a hierarchical structure. Mature cross-links enhance shear stress resistance AGE-related cross-links stiffen collagen in aged tissues Fibroblasts and myofibroblasts synthesize procollagen types I and III as a triple-helix procollagen precursor with terminal propeptides (Figure 1). These propeptides are removed by procollagen proteinases, and the collagen molecule can be incorporated

into the growing fibril. The propeptides are secreted into the blood and can be measured in the blood. Collagen propeptides can be markers of collagen synthesis if they are cleaved in each collagen molecule and if the number of propeptides detected in the circulation is proportional to the amount of collagen produced. This is the case for the procollagen type I carboxy-terminal propeptide (PICP) and probably the procollagen type I amino-terminal propeptide (PINP). There is a 1:1 stoichiometric relationship between collagen type I synthesis and PICP secretion. However, in the processing of procollagen type III to collagen type III, the carboxy-terminal and amino-terminal propeptides of collagen type III (PIIICP and PIIINP, respectively) are not completely cleaved, remaining partially in the final fiber and therefore also released during fiber breakdown. Therefore, there is some latitude in the stoichiometric relationship between the amount of type III collagen synthesized and the amount of PIIICP and PIIINP released (Ostrowska-Podhorodecka et al.,2023).

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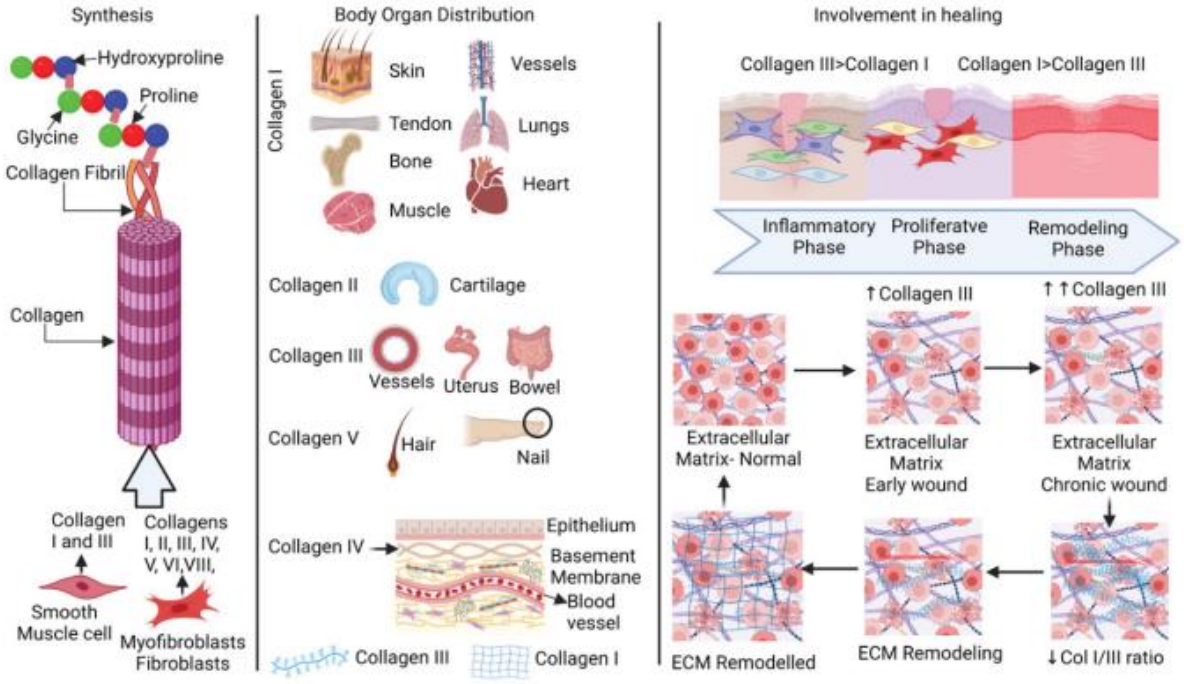


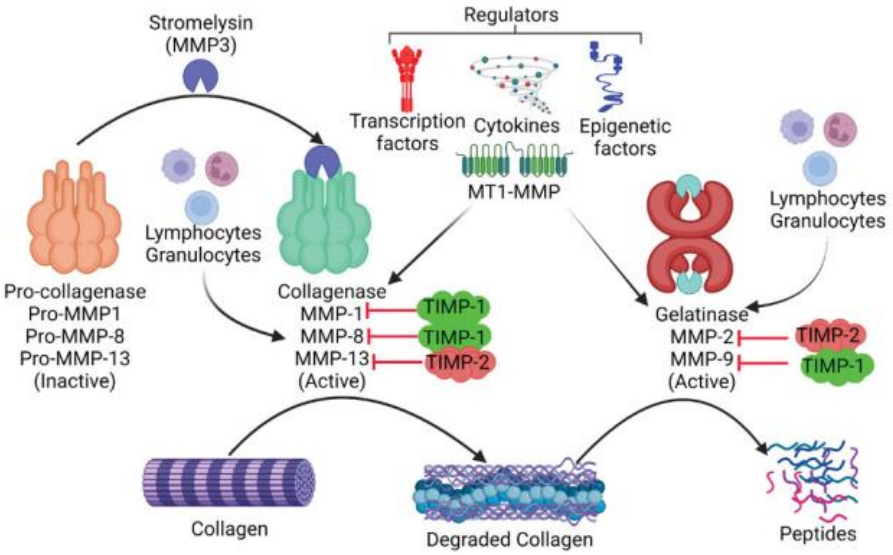

Figure 1: Collagen metabolism, deposition, and ECM turnover. Crosslinking of proline, hydroxyproline, and glycine Crosslinking between the amino acids proline, hydroxproline and glycine leads to collagen fibrilsetnogean Collagen is biosynthesized by linking toge

- Collagen Breakdown

Collagen degradation is involved in inflammation, angiogenesis, and re-epithelialization, which are regulated by complex molecular mechanisms. In the inflammatory phase, soluble collagen fragments recruit immune cells (macrophages) that scavenge the wound for microorganisms and dead tissue. This allows for the transition to the proliferative phase In this phase, collagen fragments serve as potent angiogenic cues.

Collagen also stimulates keratinocyte migration, which helps in wound re-epithelialization. Extracellular and intracellular mechanisms regulate degradation Membrane-bound and secreted proteases participate in the extracellular mechanism Intracellular mechanism involves internalization of intact collagen fibrils and degraded collagen (phagocytosis, macropinocytosis, or endocytosis) and enzymatic degradation. Pathological conditions like fibrosis result from abnormalities in the regulated turnover of collagens. Proteolytic enzyme activity during different stages of tissue healing directs the remodeling of healed tissue MMPs and serine proteases are the two main families of proteases. Their production and release are regulated and associated with specific cell types. Collagenases and gelatinases, which degrade intact and denatured fibrillar collagen, respectively, are MMPs involved in collagen turnover during tissue repair MMP-1 (collagenase-1) and MMP-8 (collagenase-2) cleave collagens I and III, while MMP-9 (gelatinase) degrades collagen IV (Figure 2). Collagenolytic enzymes recognize, bind, unwind, and cleave the individual strands of the triple helix, as has been well studied. This high specificity is believed to be due to the primary and supersecondary structure of collagen. MMPs are involved in physiological (development and tissue repair) and pathological (tumorigenesis and metastasis) processes. They also facilitate the release of bioactive fragments (matricryptins) from full-length collagens, such as endostatin and tumstatin. These bits accurately guide blood vessel pruning, enabling tissue architecture to be rebuilt during healing. Neutrophil elastase, a serine protease, also participates in this process. Thus, tissue injury and repair need a fine balance of enzyme activity and inhibition. Alterations in these enzyme levels can result in critical diseased conditions. Chronic wounds are

complicated by wounds infected with collagen-degrading enzyme-producing bacteria. MMP enzymes are key players in collagen fiber breakdown and can be inhibited by binding to tissue inhibitors of metalloproteinases (TIMPs) (TIMP-1 to TIMP-4) (Figure 2). Collagen degradation is initiated by cleavage of the peptide bond after a glycine residue approximately three-quarters of the way from the amino-terminal end of the collagen molecule by interstitial collagenase (MMP-1), neutrophil collagenase (MMP-8), and collagenase-3 (MMP-13). MMP-1 cleaves collagen type I, releasing a one-quarter carboxy-terminal telopeptide (CITP) that circulates in the blood unprocessed by the immune system. The quantity of fibrillar collagen degraded is proportional to the amount of CITP released into the circulation, with a stoichiometric ratio of 1:1. Therefore, CITP can be used as an indicator of MMP-1-dependent collagen type I degradation. MMP-2 and MMP-9, gelatinases, degrade the amino-terminal telopeptide fragment released by MMP-1 from the collagen molecule Matrikines, the matrix-derived peptides produced by these enzymes, have biological activities in the regulation of collagen metabolism and angiogenesis. Collagen type I tripeptide glycyl-histidyl-lysine (GHL) is one of them that stimulates collagen synthesis in fibroblasts. There is a redundant and synergistic function between some MMPs and matrikines, since GHL has been shown to enhance MMP-2 production and secretion by cultured fibroblasts (Singh et al.,2023).



### ❖ Relevance of Col I and Col III Ratio and Its Metabolic Control

The major regulators of collagen accumulation in tissue and cells (e.g., fibroblasts) are MMP's, the tissue inhibitors of metalloproteinases (TIMP), also necessary for stability of ECM. MMPs are the proteolytic enzymes that degrade the ECM proteins; TIMPs are the MMP-inhibitor regulating both production and degradation. The modification of the regulated synthesis and degradation of collagen has been implicated in numerous diseases, such as diverse cardiovascular disorders.

ECM includes an emulsion network, a basement membrane, proteoglycans and fibrous proteins including the fibronectins, collagens, elastins, fibrillins and laminins. They work in an integrated manner to maintain structurally integrity and stability of border cells. Similarly, the ECM has been reported to act as a transmitter of necessary chemical signals for proper tissue development.

ECM remodeling is a complex constellation of molecular, cellular and interstitial events arising at the clinical level as alterations in size, mass, shape and function of the heart following an insult. Such a process can be caused by inflammation, ischemia and cell movement or else. The changes of the 3D network configuration even may result in a structural or functional damage on cardiac ventricles, which can take place due to disturbances in collagen metabolism and subsequent defects with remodelling of the protein-based networks (Govindaraju et al.,2019).

Collagen accumulation may occur when synthesis of collagen exceeds its breakdown. The contribution to ventricular hypertrophy and diastolic dysfunction of repair- and

damage-induced myocardial fibrosis are distinct. On the other hand, ventricular dilatation and systolic dysfunction could occur depending on whether patients lose collagenous scaffolding and/or matrix as a result of increased cellular activity. These 2 patterns may occur simultaneously to some extent within a single myocardium, depending on the age of disease process and the distribution (diffuse or focal) of injury.

The effects of the collagen changes in response to pressure overload (e.g., ischemic heart disease), volume overload, and intrinsic myocardial disease or cardiomyopathy have been postulated. While combining keratinocytes in collagen matrix has also been shown to decrease inflammation and encourage normal epidermalization. This is taken to imply that collagen supports cell migration through the matrix, leading to a fast wound healing response. Fig.3 depicts phases of an injury and wound healing process during which the ratio of Col III:Col I is elevated (El Hajj et al.,2018).

## ❖ Col I/Col III Ratio in Skin and Wound

Collagen enhances tissue mechanical strength and elasticity and acts as a natural substrate for cell adhesion, proliferation, and differentiation. Biofilm-induced MMP-2 upregulation by microRNAs in the wound establishes a collagenolytic environment, drastically reducing the collagen I/collagen III ratio and weakening the biomechanical properties of the healed skin, which may render the healed skin susceptible to wound recurrence.

A recent collagen structure and function mapping study showed that in normal, unwounded tissue, the collagen fibril is in a closed conformation that, when exposed to blood after injury, exposes cell- and ligand-binding sites that may promote wound healing. Recent studies elaborate on the functions of collagen in skin and wounds.

Some studies have shown that alterations in collagen protein composition are primarily marked by a significant alteration in the type I to type III collagen ratio. The ratio of type I and type III collagen fibers is important in the regulation of fibrillogenesis and final fibril diameter and bundle structure. Pathophysiologically, mature type I collagen is responsible for mechanical stability, whereas type III collagen forming thin fibrils is mostly juvenile collagen of the early wound healing phase.

- Temporal Relationship Between Collagen Types I and III During Wound Healing

Wound healing is a complex and tightly regulated biological phenomenon, which recovers the integrity of damaged tissue. This process is a sequence of cellular and molecular events that take place in parallel, but at different times, to include the inflammatory (inflammatory phase), proliferation (proliferative phase) and remodeling phases. Among these phases, the formation and turnover of extracellular matrix (ECM) is crucial in which collagen is the most abundant structural protein. Type I and type III collagens play a crucial role in the healing of skin tissue due to their function in both

mechanical strength and structural organization of new tissue. Characterizing their temporal expression profile in the course of wound healing is essential to recognizing quality and effectiveness of tissue regeneration.

- Phase I: Inflammation and the Early Deposition of Collagen

The inflammatory and immune cell recruitment phase of wound healing, which begins soon after injury (typically during the first few days), involves inflammatory responses to the site of injury. Collagen accumulation is low at this stage. Instead, the focus of healing is to clear off the debris, infection control and wound bed priming for tissue rendering. Before this, however, fibroblasts enter the wound as they are attracted by cytokines and growth factors e.g. TGF-β (transforming growth factor-beta), PDGF (platelet derived growth factor) or VEGF (vascular endothelial growsth factor).

Type III collagen is the first detected in this acute phase. It acts as a provisional matrix during tissue granulation and supports adhesion and migration of cells. The main synthesisers of type III collagen are believed to be fibroblasts and myofibroblasts, and it is noted for its delicate fibrillar structure with flexibility rather than tensile strength. Early predominant type III collagen predominance represents a provisional, compliant matrix (substrate) promoting cell proliferation, angiogenesis, and granulation tissue formation.

The mRNA expression of type III collagen is reported to increase sharply in the first 24–72 hours following injury, before upregulation of genes coding for type I collagen [6]. Immunohistochemistry performed on early wounds usually demonstrates that there

is heavy staining for type III collagen in the vicinity of the wound edges and also within granulation tissue under an initial fibrin layer. Type I collagen, on the other hand, is relatively low at this time of development because it cannot be synthesized in a juvenile matrix and becomes elaborated subsequent to matrix remodeling (Zitnay et al.,2020).

- **Proliferative phase: granulation phase and maturation of collagen**

The proliferative stage, which generally occurs between day 3 and day 10 following injury, is characterized by a peak of fibroblast proliferation, angiogenesis and the synthesis of ECM. At this stage, collagen synthesis increases greatly and matrix changes. The most abundant isoform produced in the early proliferative stage of type III collagen will provide the frame work for replacement tissue. Yet, as fibroblasts differentiate into myofibroblasts and the granulation tissue matures, the production of type I collagen tends to increase progressively.

Type I collagen with thicker and more rigid fibers gradually replaces the type III collagen. This change is indicative of a transformation from soft, pliable matrix to one with increased tensile strength and rigidification. The augmented deposit of type I collagen is associated with decreased vascularity and cellularity because the wounded tissue changes from a regenerative to a remodeling phase of repair (Das et al.,2019).

At this stage of the healing process, at the IH level (when collagen III is organized as widely diffused in extracellular matrix), immunohistochemical studies show that there is co-localization, to a great extent, of both types of collagen in the periwound and wound bed and while type I appears stratify (as fibrillar structures) more like tissue

structure from healthy skin. The ratio of Col I/Col III starts to increase, indicating that the tissue is in the maturation process. That proportion is a critical factor for the quality of a wound, with timely turnover leading to appropriate remodeling and late replacement of type III collagen by type I resulting in scarring or ulcers that never heal (Das et al.,2019).

- Late Phase: Remodeling and Scar End Stage Formations

The remodeling phase can be several weeks to months, varying with the type and size of injury. The wound remodels to a great extent during this stage, characterized by the slow reduction in cellular activity and vascularization. A provisional ECM, type III-collagen rich ECM is the replaced by and remodeled to form a matrix highly enriched in type I collagen.

Fibroblasts and myofibroblasts are critical in this stage by secreting type I collagen and aligning and cross-linking the fibrillar network along the lines of tension of the tissue. Enzymes including lysyl oxidase participate in the production of sturdy covalent crosslinks, which increase tensile strength of new tissue. Concurrently matrix metalloproteinases (MMPs) and their natural inhibitors (TIMPs) control degradation and remodelling of the collagen network to maintain a correct balance between synthesis and degradation (Das et al.,2019).

During late remodeling, immunostaining normally shows a low percentage of type III collagen- and a densely packed well organized assembly of type I collagen fibers. The Col I/Col III ratio is maximal at this point, often even higher than in normal skin which

accounts for reduced distensibility of scar tissue when compared to unwounded dermis. In some patients excessive type I collagen deposition with abnormal fiber orientation results in development of hypertophic scars or keloids. On the contrary, insufficient collagen remodeling can result in wound dehiscence or chronic ulceration (Das et al.,2019).

- Temporal Relationship and Biological Significance

The spatial organization of the distribution of collagen types throughout healing is not only a reflection about it but also influences its result. The timely switch from type III to type I collagen synthesis is crucial in generating a scar that has good tensile strength as well as being functionally integrated with the adjacent tissue. Time delay of impaired collagen remodeling is diabetes, infection and oxidative stress and also poor vascular supply that disturb fibroblast function and collagen cross-linking.

The control of this temporal patterning is carried out by multiple signalling pathways, including the TGF-β and connective tissue growth factor (CTGF) reigns, and different integrins which sense mechanical force. They synchronize the induction of collagens, activation of fibroblasts and remodeling of ECM. Experimental wound healing models also show that tension or mechanical loading across a wound can promote the transition from type III to type I collagen production, indicating a role of biomechanical stimuli in collagent organization (Das et al.,2019).

- Temporal Visualization through Immunohistochemistry

Immunohistochemical analysis provides a valuable approach to visualize and quantitatively address the temporal regulation of types I, III, IV collagens in wound healing. The localization of such proteins at post-injury time-points can be mapped using specific antibody probes designed against collagen I and III. Early-stage specimens usually exhibit type III staining diffusely with little such presence of type I. Intermediate samples show co-localization of the two types in the granulation tissue, whereas late-stage samples demonstrate intense type I staining was focused on the remodelled scar tissue.

Analysis of immunohistochemical sections by quantitative image analysis permits determination of the Col I/Col III ratio with time and offers an objective measure for wound maturity. It also provides a means to assess the effectiveness of therapeutic interventions, and drugs including growth factors, stem cells or biomaterials on the dynamics of collagen remodeling (Das et al.,2019).

❖ **Immunohistochemical Evaluation**

Immunohistochemical (IHC) staining is one of the principal techniques used to analyze the expression and distribution of proteins in tissue. For wound healing, therefore, IHC offers a potent tool to study the temporal and spatial profiles of collagen types I and III, the dominant/stabilising fibrillar collagens having profound mechanical/structural significance in skin. Immunohistochemistry, by combining morphological imaging with molecular identification, supply between tissue structural readout and amounts

signal quantification, is a powerful tool to analyze changes in ECM (extracellular matrix) at the time of wound healing (Khalaf et al.,2019).

- Principles of Immunohistochemistry

Immunohistochemistry depends on the selective binding of an antibody to its specific antigen present in a section of tissue. In this study, antibodies specific for collagen type I (Col I) and collagen type III (Col III) are used to demonstrate the production and topographic distribution of these macromolecules in wounded and unwounded skin. The antigen-antibody reaction is specific which makes it possible to map the exact localisation, the intensity and the organization of the collagens at various stages of healing. Immunohistochemical detection of these bound antibodies is usually performed by chromogenic stains or fluorescent markers which give rise to a visible signal that can be detected with standard light or fluorescence microscopy (Khalaf et al.,2019).

Color is deposited at the antigen site following generation of a chromogenic reaction through use of an enzyme-conjugated secondary antibodies (typically horseradish peroxidase or alkaline phosphatase). The color reaction degree and location is linearly related to the content and distribution of target protein molecules. Or fluorescent labeling offers higher resolution and multiplexing, in which multiple antigens can be visualized at the same time. In wound healing studies, chromogenic (eg: diaminobenzidine - DAB) detection is frequently employed due to its stability and compatibility with routine light microscopy (Kamar et al.,2019).

- Tissue Preparation and Antigen Retrieval

There are stringent requirements for tissue processing and handling to ensure the correctness of immunohistochemistry results. In wound-healing experiments, skin biopsies are obtained at multiple time points after wounding to sample expression patterns of collagen as they change following injury. Specimens are fixed in neutral buffered formalin, although the use of other fixatives is acceptable so long as tissue morphology and protein antigenicity can be preserved. Following fixation, tissues are dehydrated through alcohols, cleared in xylene and embedded in paraffin blocks for sectioning. Thin slices usually 4–6 µm thick are cut and adhered to adhesive coated glass slides before undergoing a staining process.

Epitope unmasking is frequently required to draw out the antigenic sites as formalin fixation may cross-link and so hide them. This process may include heat-mediated epitope retrieval (HIER) with citrate or EDTA buffer, or digestion by proteolytic enzymes such as trypsin and pepsin. The preferred method also relies on the nature of the antigen and antibody. Good antigen retrieval increases staining quality and reproductibility (Kisling et al.,2019).

- Blocking and Antibody Incubation

To reduce background and non-specific binding of antibodies, tissue sections are blocked first. This is usually normal serum or bovine serum albumin (BSA) that fills specific binding sites. In addition, endogenous peroxidase activity is mitigated by

hydrogen peroxide to avoid false positive findings with chromogenic detection systems.

Sections are incubated with the primary antibody (collagen type I and type III, respectively) for a determined time (1 h at room temperature up to overnight at 4°C depending on the affinity of the antibody and tissue). After washing to remove unbound antibodies, sections were incubated with the secondary antibody bound to an enzyme or fluorophore which binds to the species of origin of the primary antibody. The end signal results from reaction of chromogenic substrate or by direct fluorescent detection (Kisling et al.,2019).

- Visualization and Microscopic Examination

In the chromogenic detection, the horseradish peroxidase (HRP)-linked secondary antibodies mediated conversion of substrate DAB to a brown precipitate indicating site of presence of antigen. Sections are then lightly counterstained with hematoxylin to demonstrate nuclei and tissue structure. Collagen I or III positive areas are brown stained, and can be marked either golden-brown (collagen I) or dark brown (collagen III). When carrying out serial sections staining of collagen I and collagen III one is able to distinguish between the two types of collagens.

Distribution of collagen types are then qualitatively and quantitatively examined. Adaptation "issue" Qualitatively, the researcher evaluates the intensity and pattern of staining (and its localization) in various wound zones, including the wound edge, granulation tissue and newly-formed dermis. For quantification, the optical density or

positive straining percent area can be measured by using an image analyzing software (e.g., ImageJ or Aperio). These quantitations objectively capture changes in collagen expression at various time points during dynamic matrix remodeling (Kamar et al.,2019).

- Temporal Immunohistochemical Findings

Collagen I and III predominately exhibit weak staining (day 1–3 post-injury) in the early phases of wound healing, with type III showing slightly more intense staining at the wound edge. This is the same as that of fibroblast initiation activation and early extracellular matrix deposit o ion. In the proliferative phase (5--10 days), there is strong immunoreaction of type III collagen in granulation tissue, reflecting its predominance as a major component of the early matrix scaffold. The staining of type I collagen starts to increase mainly in the deep dermal layers and around the wound (Kamar et al.,2019).

At the remodelling phase (pooled 2 weeks), the pattern shifts significantly. Staining is weaker and more localized (for type III collagen), while it's replaced by crisper type I collagen, which appears as dense thick-walled structures across the wound bed. The increasing Col I/Col III ratio, reflected by differential staining intensities, reflects the change from a soft supple provisional matrix to a stiff mature scar tissue. Late-stage samples might produce thick, parallel type I collagen bundles with less type III surface expression, which indicated formation of a mechanically robust but also less elastic scar (Kamar et al.,2019).

- Spatial Localization Patterns

Immunohistochemistry further demonstrates that the two types of collagen have different spatial distribution. Type III collagen is found around neoformed capillaries as well as in the loose connective tissue of granulation zones, pointing to its relationship with flexibility and angiogenesis. By comparison, type I collagen distributes in the reticular dermis and in proximity of tensile areas to provide structural resistance. This spatial pattern of deposition implies functional specificity, such as the contribution of type III collagen to cell infiltration and remodeling and that of type I collagen to the permanent architecture of repaired tissue (Khalaf et al.,2019).

- Quantitative Analysis and Image Scoring

Immunohistochemical results are frequently quantified for statistical interpretation by semi-quantitative scoring systems or computerized image analysis. Semi-quantitative score is determined by the staining intensity (0, negative; 1, mild; 2, moderate; and 3, severe) multiplied % of positive in each field. The product of the intensity and percentage score yields a total immunoreactivity score (IRS), which provides an objective measure for comparison among samples.

Instead, collagen deposition can be quantified by analyzing digital images for pixel intensity and area fraction. This quantitative method reduces observer bias and improves the reproducibility. Under histologic correlation, these measurements provide important kinetic information on collagen turnover and maturation in wound tissue (Khalaf et al.,2019).

- Significance and Interpretation

Immunohistochemical examination of collagens I and III gives a visual and quantitative picture of remodelling extracellular matrix during wound healing. It validates that collagen III becomes the prominent type in an early phase of the wound, thereby promoting rapid tissue regeneration and flexibility, while subsequently collagen I is best expressed for tensile strength and fine structural integrity. The change in their ratio is the natural course of going from a granulation phase to one of scarring.

Dysregulation in IHC staining including continuous expression of CI and abnormal overdeposition of CIII may be indicative of chronic healing, fibrosis, and hypertrophic scarring. Accordingly, immunohistochemical analysis is not only descriptive but also diagnostic and it permits to determine the influence of therapeutic substances, biomaterials or growth factors on wound healing (Kisling et al.,2019).

Conclusions

The distribution of collagen types I and III in wound healing was assessed over time and space by immunohistochemical analysis in the present study. Taken together, the results indicate that collagen remodeling throughout tissue healing is a dynamic and tightly regulated activity, which mirrors the temporal synchronization of fibroblasts, inflammatory cells and extracellular substrates. The temporal and spatial relationship in the expression of collagens type I vs. III is a reflection on the essential biological process of wound maturation and scar formation.

At the early inflammatory phase of the wound healing process, immunohistochemical analysis revealed a small amount of deposited collagen in which type III collagen was predominantly located at around the edges and granulation tissue. This observation suggests that collagen III is highly synthesized during the early ECM and serves as provisional scaffold for migrating fibroblasts and endothelial cells. Loose and fine fibrillar structure of type III collagen contributes to the flexibility of tissues, cellular infiltration, and angiogenesis as well as early tissue regeneration.

In the proliferative phase, type III collagen became strikingly predominant throughout the granulation tissue, and type I collagen developed in the deeper dermal layers. During this time, the presence of both collagens is indicative of a shift from an initially transient and compliant matrix to that which will ultimately become organized and stable connective tissue. It is important that the two forms of collagen remain in equilibrium to allow for correct wound contraction and tensional strength. Overproduction or retardation of production of either one can result in healing problems, including hypertrophic scar and persistent wound.

In the remodeling/maturing phase a complete change was registered with collagen type I being the most represented structure in reepithelialized tissue. Type I collagen fibers are arranged densely and in parallel (b), suggesting higher mechanical strength compared to the previously type III-rich granulation tissue. The progressive decrease of type III collagen and increase in type I collagen indicates maturation of extracellular matrix and the termination of healing process. The immunohistochemical data

therefore support the conventional collagen composition change in normal wound healing—from type III–enriched to type I–predominant ECM.

On a spatial basis, the collagen type III was predominantly limited to areas of neovascularisation and active fibroblast proliferation, whereas after 2 days the type I collagen lay more deeply within the dermis and ran parallel with tensile forces. This spatial separation underlines their functional interplay: typeIII confers plasticity and structural guidance to organize the cells within the tissue, while typeIac meninges confer long-term mechanical integrity to the tissue.

The study as a whole highlights the importance of immunohistochemistry in being an accurate technique in terms of the detection and quantification of collagen deposition. This allows for the visualization of protein distribution in a tissue's structure and qualitative, quantitative information about molecular events occurring within healing. An appreciation of these temporal and spatial variations is important not only in defining the normal sequence of physiological repair but also as a baseline to evaluate pathology, or therapeutic manipulation designed to improve wound healing.

In summary, during the proliferative phase, CoIII establishes an initial structural scaffold encouraging cellular infiltration and matrix organization followed by replacement with CoI resulting in development of tensile strenght and physical tranquality during remodeling. The directed switches between these two collagen types, as detected immunohistochemically, outline the basic chronology of wound maturation. Shifts in this temporal-spatial balance can lead to failure of healing or

unwanted scarring, underscoring collagen metabolism as a key element to target for coming therapeutics and regenerative approaches.

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