STUDY OF FIBROBLAST COUNT FOR AGE ESTIMATION OF CUTANEOUS WOUNDS

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DISCLAIMER

I hereby declare that the work in this dissertation is my on except for quotation and summaries which have been duly acknowledged.

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31st DISEMBER 2008 **DR NUR AYUTIMASERY**

DEDICATION

To my beloved husband, Mohd Noor Aziron b. Kassim

To my three lovely children, Muhammad Aliff Syafiq, Muhammad Afiq Syarif and Muhammad Afif Syahir

Your love, patience, support and do'a made this dissertation possible.

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ABSTRACT

The determination of wound age as well as the distinction between antemortem and postmortem wounds, has been one of the most important medico legal interest. Since 1867 the issue has been raised and has lead to numerous researches to determine the age of the cutaneous wound based on histological markers. This study analyzes the use of fibroblast as a marker to estimate the abrasion wound age.

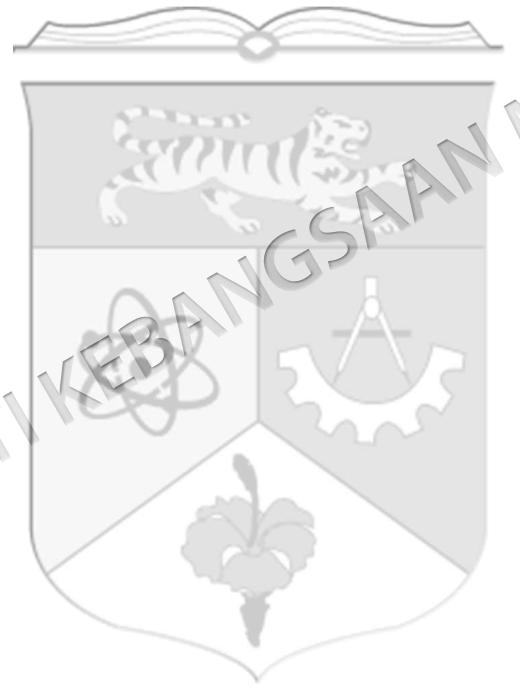
Objectives: To determine the wound age by using fibroblast as a marker which is identified by Hemotoxylin & Eosin (H&E) staining and the aid of immunohistochemical study of basic Fibroblast Growth Factor (bFGF).

Subjects and Methods: 20 adult skin samples which display abrasion wounds were taken from the body which had sustained the abrasion wounds, admitted to the ward for treatment for various durations, however succumbed to the illness and subsequently undergone medico-legal autopsy. The samples were stained with immunohistochemical of basic Fibroblast Growth Factor (bFGF) and the fibroblasts were identified by the morphology and reactivity to the stain. The identified fibroblasts were counted in 10 high power fields of each sample and scored using 3-tier system as follows : 10-50 fibroblasts, >50-100 fibroblasts and > 100 fibroblasts.

Results: Wounds age <3 days displayed 10-50 fibroblasts per 10 high power fields, wounds age 3-7 days showed marked increase in the number of fibroblast (>100 fibroblasts) and the number of fibroblasts in wound age > 7 days were decreasing to 50-100 fibroblasts.

There was significant correlation between the number of fibroblast and the age of the wound ($p<0.0001$).

Conclusions: This study has shown that the number of fibroblast displayed a schema in wound healing and wound age. Therefore fibroblast is a useful marker to estimate the cutaneous wound age.



ABSTRAK

Menentukan umur sesuatu kecederaan dan juga menentukan samada sesuatu kecederaan itu dialami sebelum atau selepas kematian telah menjadi salah satu bidang medico-legal yang menarik. Sejak tahun 1867, isu ini telah diketengahkan dan telah melahirkan banyak kajian bagi menentukan umur sesuatu luka pada permukaan kulit menggunakan penanda histologi. Kajian ini menganalisa penggunaan fibroblast sebagai penanda dalam menentukan umur kecederaan abrasi pada kulit.

Objektif: Bagi menganggarkan umur kecederaan dengan menggunakan fibroblast sebagai penanda, yang mana dikenalpasti melalui kaedah pewarnaan Hemotoxylin & Eosin (H&E) dan immunohistokemikal yang menggunakan 'basic Fibroblast Growth Factor (bFGF).

Subjek dan kaedah: Sejumlah 20 sampel kulit orang dewasa yang mempunyai kecederaan abrasi diambil daripada jasad yang mengalami kecederaan tersebut, dirawat di hospital bagi tempoh tertentu, tetapi malangnya meninggal dunia kerana penyakit yang dialami dan seterusnya terpaksa dibedah siasat secara medikolegal. Sampel-sampel tersebut diwarnakan secara immunohistokemikal iaitu basic Fibroblast Growth Factor (bFGF) dan fibroblast dikenalpasti melalui morpologi dan tindakbalas positif dengan pewarnaan tersebut. Fibroblast yang telah dikenalpasti itu dikira di dalam 10 bidang berkuasa tinggi di dalam setiap sampel dan diberi pemarkahan menggunakan sistem 3 lapis iaitu : 10-50 fibroblast, >50-100 fibroblast dan > 100 fibroblast.

Keputusan: Kecederaan berumur <3 hari menunjukkan 10-50 fibroblast dalam 10 bidang berkuasa tinggi, kecederaan berumur 3-7 hari menunjukkan peningkatan mendadak jumlah fibroblast iaitu >100 fibroblast dan jumlah fibroblast dalam kecederaan berumur >7 hari adalah menurun kepada 50-100 fibroblast. Didapati terdapat hubungkait yang signifikan diantara bilangan fibroblast dan umur kecederaan (p<0-0001).

Kesimpulan:

Kajian ini menunjukkan bilangan fibroblast mempamerkan skema yang tertentu di dalam proses penyembuhan luka dan umur luka. Dengan ini kajian ini menunjukkan fibroblast boleh digunakan sebagai penanda dalam menentukan umur kecederaan pada kulit.

CHAPTER 1

INTRODUCTION

Wound examination is indispensable in forensic practice. It is always necessary to determine wound vitality or wound age to correctly evaluate the relationship between death and any wounds. Wound age estimation based on the evolution of wound healing has been an area of interest in forensic pathology since 1867, thus, the determination of wound vitality or wound age is a classic but still modern theme in forensic pathology.

Histological determination of wound age

Dating of wounds by histology and histochemistry has been actively pursued in view of the histology of wound healing generally follows a definite order as so the wound aging. In wound healing, a set of complex biochemical events takes place in a closely orchestrated cascade that serve to eliminate the injurious agents, contain the damage, and prepare the surviving cells for replication. These events overlap in time and may be categorized into separate steps: the inflammatory, proliferative, and remodeling phases[1] (Fig. 1).

Inflammatory phase

In the inflammatory phase (lag phase/resting phase), clotting takes place in order to obtain hemostasis, and various factors are released to attract cells that phagocytise debris, bacteria, and damaged tissue and release factors that initiate the proliferative phase of wound healing [2].

Coagulation cascade

Injury to vascular tissue initiates the extrinsic coagulation cascade by releasing intracellular calcium and tissue factor that activate factor VII. The resulting fibrin plug achieves hemostasis aided by reflex vasoconstriction. This plug acts as a lattice for the aggregation of platelets, the most common and "signature" cell type of the early inflammatory phase[3] (Fig. 2). When tissue is first wounded, blood comes in contact with collagen, triggering platelets to begin secreting inflammatory factors. Platelets also express glycoproteins on their cell membranes that allow them to stick to one another and to aggregate, forming a mass. Injured tissues, through activated phospholipase A, simultaneously catalyze arachidonic acids to produce vasoactive prostaglandins and thromboxane, collectively known as eicosanoids. Eicosanoids mediate activity influencing platelet plug formation, vascular permeability, and cellular chemotaxis to influence wound healing. For example, thromboxane A2 mediates vasoconstriction and platelet aggregation [4].

Fibrin and fibronectin cross-link together and form a plug that traps proteins and particles and prevents further blood loss. This fibrin-fibronectin plug is also the main structural support for the wound until collagen is deposited. Migratory cells use this plug as a matrix to crawl across, and platelets adhere to it and secrete factors. The clot is eventually lysed and replaced with granulation tissue and then later with collagen [5].

Platelets

Platelets, the cells present in the highest numbers shortly after a wound occurs, release a number of chemicals into the blood, including ECM proteins and cytokines, including growth factors [1]. Platelets elaborate a number of proinflammatory substances, such as

adenosine diphosphate, tissue growth factor beta (TGF-ß), and platelet-derived growth factors (PDGF). These growth factors act on surrounding cells and stimulate chemotaxis of neutrophils, monocytes, and fibroblasts to the area of injury [3].

Platelets also release other proinflammatory factors like serotonin, bradykinin, prostaglandins, prostacyclins, thromboxane, and histamine, which serve a number of purposes, including to increase cell proliferation and migration to the area and to cause blood vessels to become dilated [6].

Vasoconstriction and vasodilation

Immediately after a blood vessel is breached, ruptured cell membranes release inflammatory factors like thromboxanes and prostaglandins that cause the vessel to spasm to prevent blood loss and to collect inflammatory cells and factors in the area. This vasoconstriction lasts five to ten minutes and is followed by vasodilation, which peaks at about 20 minutes post-wounding. Vasodilation is the result of factors released by platelets and other cells. The main factor involved in causing vasodilation is histamine. Histamine also causes blood vessels to become porous, allowing the tissue to become edematous because proteins from the bloodstream leak into the extravascular space, which increases its osmolar load and draws water into the area. Increased porosity of blood vessels also facilitates the entry of inflammatory cells like leukocyte into the wound site from the bloodstream [7].

Polymorphonuclear neutrophils

Within an hour of wounding, polymorphonuclear neutrophils (PMNs) arrive at the wound site and become the predominant cells in the wound for the first five days after the injury occurs, with especially high numbers on the second day. They are attracted to the site by

fibronectin, growth factors, and substances such as neuropeptides and kinins. Neutrophils phagocytise debris and bacteria and also kill bacteria by releasing free radicals in what is called a respiratory burst. They also cleanse the wound by secreting proteases that break down damaged tissue. Neutrophils usually undergo apoptosis once they have completed their tasks and are engulfed and degraded by macrophages [8].

Other leukocytes to enter the area include helper T cells, which secrete cytokines to cause more T cells to divide and to increase inflammation and enhance vasodilation and vessel permeability. T cells also increase the activity of macrophages [9].

Macrophages

Macrophages are essential to wound healing. They replace PMNs as the predominant cells in the wound by two days after injury. Attracted to the wound site by growth factors released by platelets and other cells, monocytes from the bloodstream enter the area through blood vessel walls. Numbers of monocytes in the wound peak one to one and a half days after the injury occurs. Once they are in the wound site, monocytes mature into macrophages, the main cell type that clears the wound area of bacteria and debris [8].

The macrophage's main role is to phagocytise bacteria and damaged tissue, and it also debrides damaged tissue by releasing proteases. Macrophages also secrete a number of factors such as growth factors and other cytokines, especially during the third and fourth post-wounding days. These factors attract cells involved in the proliferation stage of healing to the area [7]. Macrophages produce a wide variety of important substances, such as IL-1 and basic fibroblast growth factor (bFGF). IL-1 stimulates the proliferation of inflammatory cells and promotes angiogenesis through endothelial cell replication. bFGF is a chemotactic and mitogenic factor for fibroblasts and endothelial cells [10].

Macrophages are stimulated by the low oxygen content of their surroundings to produce factors that induce and speed angiogenesis and they also stimulate cells that reepithelialize the wound, create granulation tissue, and lay down a new extracellular matrix. Because they secrete these factors, macrophages are vital for pushing the wound healing process into the next phase [6]

Proliferative (Reconstruction) phase

About two or three days after the wound occurs, fibroblasts begin to enter the wound site, marking the onset of the proliferative phase [4].

Angiogenesis

Also called neovascularization, the process of angiogenesis occurs concurrently with fibroblast proliferation when endothelial cells migrate to the area of the wound. To migrate, endothelial cells need collagenases and plasminogen activator to degrade the clot and part of the ECM. Endothelial cells are also attracted to the wound area by fibronectin found on the fibrin scab and by growth factors released by other cells. Endothelial growth and proliferation is also stimulated by hypoxia and presence of lactic acid in the wound. In a low-oxygen environment, macrophages and platelets produce angiogenic factors which attract endothelial cells chemotactically [11].

Fibroplasia and granulation tissue formation

Simultaneously with angiogenesis, fibroblasts begin accumulating in the wound site. Fibroblasts begin entering the wound site two to five days after wounding as the inflammatory phase is ending [3], and they become the prominent cell type by 3 to 5 days

in clean, noninfected wounds [13]. By the end of the first week, fibroblasts are the main cells in the wound. Fibroplasia ends two to four weeks after wounding [9].

Migration of fibroblasts to the site of injury and their subsequent proliferation are triggered by multiple growth factors, including TGF-β, PDGF, EGF, FGF, and the cytokines IL-1 and TNF. The sources of these growth factors and cytokines include platelets, a variety of inflammatory cells (notably macrophages), and activated endothelium. Macrophages are important cellular constituents of granulation tissue, clearing extracellular debris, fibrin, and other foreign material at the site of repair.

These cells also elaborate TGF-β, PDGF, and FGF and there-fore promote fibroblast migration and proliferation. If the appropriate chemotactic stimuli are present, mast cells, eosinophils, and lymphocytes may also accumulate. Each of these cells can contribute directly or indirectly to fibroblast migration and proliferation.

In the first two or three days after injury, fibroblasts mainly proliferate and migrate, while later, they are the main cells that lay down the collagen matrix in the wound site. Fibroblasts from normal tissue migrate into the wound area from its margins. Initially fibroblasts use the fibrin scab formed in the inflammatory phase to migrate across, adhering to fibronectin. Fibroblasts then deposit ground substance into the wound bed, and later collagen, which they can adhere to for migration [1].

During the first week, fibroblasts begin producing glycosaminoglycans and proteoglycans, the ground substance for granulation tissue, as well as collagen, in response to macrophage-synthesized bFGF and TGF-ß, as well as PDGF.

Fibroblasts generate not only collagen molecules but also cytokines such as PDGF, TGF-ß , bFGF, keratinocyte growth factor, and insulin like growth factor-1 [3].

Granulation tissue is needed to fill the void that has been left by a large, open wound that crosses the basement membrane. It begins to appear in the wound even during the inflammatory phase, two to five days post wounding, and continues growing until the wound bed is covered. Granulation tissue consists of new blood vessels, fibroblasts, inflammatory cells, endothelial cells, myofibroblasts, and the components of a new, provisional ECM. The provisional ECM is different in composition from the ECM in normal tissue and includes fibronectin, collagen, glycosaminoglycans, and proteoglycans. Its main components are fibronectin and hyaluronan, which create a much hydrated matrix and facilitate cell migration. Later this provisional matrix is replaced with an ECM that more closely resembles that found in non-injured tissue [1].

Growth factors (PDGF, TGF-β) and fibronectin encourage proliferation, migration to the wound bed, and production of ECM molecules by fibroblasts. Fibroblasts also secrete growth factors that attract epithelial cells to the wound site [1].

Collagen deposition

One of fibroblasts' most important duties is the production of collagen. Fibroblasts begin secreting appreciable collagen by the second or third post-wounding day, and its deposition peaks at one to three weeks. Collagen production continues rapidly for two to four weeks which are cross-linked and organized into bundles. Collagen deposition is important because it increases the strength of the wound [1].

Epithelialization

The formation of granulation tissue in an open wound allows the reepithelialization phase to take place, as epithelial cells migrate across the new tissue to form a barrier between the wound and the environment. Basal keratinocytes from the wound edges and dermal

appendages such as hair follicles, sweat glands and sebaceous glands are the main cells responsible for the epithelialization phase of wound healing. They advance in a sheet across the wound site and proliferate at its edges, ceasing movement when they meet in the middle [1].Keratinocytes migrate without first proliferating. Migration can begin as early as a few hours after wounding. However, epithelial cells require viable tissue to migrate across, so if the wound is deep it must first be filled with granulation tissue. Thus the time of onset of migration is variable and may occur about one day after wounding. Cells on the wound margins proliferate on the second and third day post-wounding in order to provide more cells for migration [1].

If the basement membrane is not breached, epithelial cells are replaced within three days by division and upward migration of cells in the stratum basale in the same fashion that occurs in uninjured skin. However, if the basement membrane is ruined at the wound site, reepithelization must occur from the wound margins and from skin appendages such as hair follicles and sweat and sebaceous glands that enter the dermis that are lined with viable keratinocytes [1].

Migration of keratinocytes over the wound site is stimulated by lack of contact inhibition and by chemicals such as nitric oxide. Before they begin to migrate, cells must dissolve their desmosomes and hemidesmosomes, which normally anchor the cells by intermediate filaments in their cytoskeleton to other cells and to the ECM. Transmembrane receptor proteins called integrins, which are made of glycoproteins and normally anchor the cell to the basement membrane by its cytoskeleton, are released from the cell's intermediate filaments and relocate to actin filaments to serve as attachments to

the ECM for pseudopodia during migration. Thus keratinocytes detach from the basement membrane and are able to enter the wound bed [7].

Before they begin migrating, keratinocytes change shape, becoming longer and flatter and extending cellular processes like lamellipodia and wide processes that look like ruffles. Actin filaments and pseudopodia form. During migration, integrins on the pseudopod attach to the ECM, and the actin filaments in the projection pull the cell along. The interaction with molecules in the ECM through integrins further promotes the formation of actin filaments, lamellipodia, and filopodia.

Fibrin, collagen, and fibronectin in the ECM may further signal cells to divide and migrate. Migrating keratinocytes use the fibronectin cross-linked with fibrin that was deposited in inflammation as an attachment site to crawl across [6].

As keratinocytes migrate, they move over granulation tissue but underneath the scab, separating it from the underlying tissue. Epithelial cells have the ability to phagocytize debris such as dead tissue and bacterial matter. Keratinocytes secrete plasminogen activator, which activates plasmin to dissolve the scab. Keratinocytes also excrete collagenases and proteases like matrix metalloproteinases (MMPs) to dissolve the ECM and also dissolve the basement membrane [1]. As keratinocytes continue migrating, new epithelial cells proliferate, which normally begins a few days after wounding and occurs at a rate that is 17 times higher in this stage of epithelialization than in normal tissues.

Growth factors, stimulated by integrins and MMPs, cause cells to proliferate at the wound edges. Keratinocytes themselves also produce and secrete factors, including growth factors and basement membrane proteins, which aid both in epithelialization and in other phases of healing.

Keratinocytes continue migrating across the wound bed until cells from either side meet in the middle, at which point contact inhibition causes them to stop migrating. When they have finished migrating, the keratinocytes secrete the proteins that form the new basement membrane. Cells reverse the morphological changes they underwent in order to begin migrating; they reestablish desmosomes and hemidesmosomes and become anchored once again to the basement membrane. Basal cells begin to divide and differentiate in the same manner as they do in normal skin to reestablish the strata found in reepithelialized skin [8].

Contraction

Around a week after the wounding takes place, fibroblasts have differentiated into myofibroblasts and the wound begins to contract. In full thickness wounds, contraction peaks at 5 to 15 days post wounding. Contraction can last for several weeks and continues even after the wound is completely reepithelialized. Contraction occurs in order to reduce the size of the wound. Myofibroblasts are attracted by fibronectin and growth factors and they move along fibronectin linked to fibrin in the provisional ECM in order to reach the wound edges. They form connections to the ECM at the wound edges, and they attach to each other and to the wound edges by desmosomes. Also, at an adhesion called the fibronexus, actin in the myofibroblast is linked across the cell membrane to molecules in the extracellular matrix like fibronectin and collagen, which allow them to pull the ECM when they contract, reducing the wound size [12]

Maturation and remodeling phase

During Maturation, type III collagen, which is prevalent during proliferation, is gradually degraded and the stronger type I collagen is laid down. Collagen fibers are rearranged, cross-linked, and aligned along tension lines. As the phase progresses, the tensile strength of the wound increases, with the strength approaching 50% that of normal tissue by three months after injury and ultimately becoming as much as 80% as strong as normal tissue [9].

The role of basic Fibroblast Growth Factor in determination of wound age

With the development of immunohistochemistry and chemical analyses, the scientific field of wound age determination has advanced progressively during recent years. In particular, it has been demonstrated that esterase, ATPase, acid phosphatase and alkaline phosphatase have all been reported to increase during the early phases of cutaneous wound healing [13]. The proinflammatory cytokines, interleukin-1beta (IL-1ß), interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α) can serve as a useful tool for the estimation of vitality and wound age, in particular in early post traumatic prior to leucocyte reaction [14]. Immunohistochemical studies on IL-8, MCP-1, MIP-1α [15] and melanocyte migration into human scar tissue were performed, and the mRNA dynamics of inflammatory cytokines, such as IL-1α, IL-1β, IL-6, TNFα and IL-10 were examined for dermal wound age determination. It has also been demonstrated that collagens, cytokines, and growth factors are useful candidates and markers for the determination of wound vitality or age. Recently, it was shown that basic Fibroblast Growth Factor (bFGF) stimulated wound healing in impaired mice and a positive regulator in angiogenesis [16]. Therefore, bFGF was considered to play an important role in wound healing. According to Takamiya, 2003, it was demonstrated that bFGF protein was induced in early phase of dermal healing, therefore suggested that bFGF could be a useful marker as a dermal wound age determination [17].

Fibroblast Growth Factor (FGF).

This is a family of growth factors containing 22 (human) members [22], of which acidic FGF (aFGF, or FGF-1) and basic FGF (bFGF, or FGF-2) are the best characterized. FGF-

1 and FGF-2 are made by a variety of cells. Released FGFs associate with heparan sulfate in the ECM, which can serve as a reservoir for storing inactive factors. FGFs are recognized by a family of cell-surface receptors that have intrinsic tyrosine kinase activity. A large number of functions are attributed to FGFs, including the following [18]:

- New blood vessel formation (angiogenesis): FGF-2, in particular, has the ability to induce the steps necessary for new blood vessel formation both in vivo and in vitro (see below).
- Wound repair: FGFs participate in macrophage, fibroblast, and endothelial cell migration in damaged tissues and migration of epithelium to form new epidermis.
- Development: FGFs play a role in skeletal muscle development and in lung maturation. For example, FGF-6 and its receptor induce myoblast proliferation and suppress myocyte differentiation, providing a supply of proliferating myocytes. FGF-2 is also thought to be involved in the generation of angioblasts during embryogenesis. FGF-1 and FGF-2 are involved in the specification of the liver from endodermal cells.
- Hematopoiesis: FGFs have been implicated in the differentiation of specific lineages of blood cells and development of bone marrow stroma.

Expression of basic fibroblast growth factor has been studied and it has been found on most of the positive cells were fibroblast but it was also expressed in macrophages [19]. In injured skin wound, the expression was detected in epidermal cells, fibroblasts, and endothelial cells in intact skin, and in neutrophils it became detectable at 24 and 72 h post-injury [17].

1.3 OBJECTIVES

1.3.1 General objective

To estimate the age of skin wounds by using fibroblast as a marker.

1.3.2 Specific objectives

1. To count the fibroblast number seen on histology section of abrasion wounds using immunohistochemical study of basic Fibroblast Growth Factor and correlate it with the wound age.
2. To evaluate the use of fibroblast as a marker for cutaneous wound age estimation

1.4 RESEARCH HYPOTHESES

There is a significant correlation between fibroblast counts with the age of cutaneous wound.

CHAPTER 5

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