CHAPTER II

THEORY

2.1 Skin [18,19]

Structure of skin

Skin is the largest human organ. It covers between 1.5 and 2 m^2 , comprising about one sixth of total body weight. It consists of three layers, which are epidermis, dermis, and subcutaneous tissue as shown in Figure 2.1.

Epidermis

The outer thinner layer known as epidermis is composed mainly of epithelial cells. The outermost cells contain protein keratin and are known as keratinocytes. The basal or deepest epidermal cells are anchored to basement membrane by adhesion molecules (or glue), namely fibronectin. These immature cells are continually dividing and migrating toward skin surface to replace lost surface cells. The same type of regenerating epidermal cells are found in hair follicles and other skin appendages which are anchored in the dermis. As mature cells migrate to the surface, they form keratin which becomes an effective barrier to environmental hazards such as infection and excessed water evaporation.

Replacement of the epidermal layer by this regenerative process takes 2-3 weeks. Cues and biologic stimuli at wound surface are necessary to direct proper orientation and mitotic response of epidermal cells. Many cues come from dermal elements, especially matrix proteins and matrix glycosaminoglycan. Characteristics of epidermis are protection from environmental insults, ability to regenerate every 2-3 weeks resulting from biologic cues provided by dermis, basement membrane.

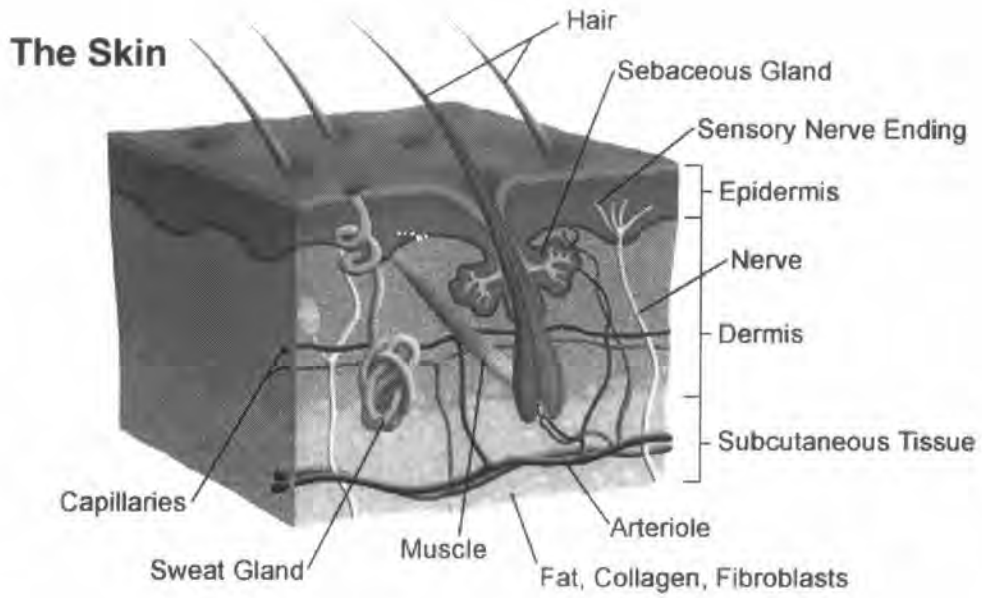

Figure 2.1 Anatomy of skin [18]

Dermis

The middle layer of skin is called dermis which is a non-regenerative organ. Dermis is a very dynamic layer of thick connective tissue, also in constant turnover. Dermis is divided into a thin superficial layer known as papillary dermis containing anchoring epidermal rete pegs and thicker deeper portion known as reticular dermis. Papillary dermis is the major factory for proteins providing direction for epidermal replication. Upper dermis also contains the highest blood flow. The primary cell type is fibroblast which produces the key structural extracellular matrix protiens, collagen and elastin, as well as matrix or ground substance.

In addition, these cells produce key adhesion proteins used to attach epidermal cells to basement membrane. Fibronectin is a key fibroblast derived signal protein for orchestration of healing. The ground substance or matrix is made up of polysaccharide protein complex known as glycosaminoglycan or GAG component as well as hyaluronic acid. The matrix provides a semi-fluid which allows for cell and connective tissue orientation as well as nutrient diffusion to cells and a scaffolding for cell migration.

Subcutis (hypodermis)

The subcutis refers to fat tissue below the skin. It consists of spongy connective tissue interspersed with energy-storing adipocytes (fat cells). Fat cells are grouped together in large cushion-like clusters held in place by collagen fibres called connective tissue septa or sheaths.

The subcutis is heavily interlaced with blood vessels, ensuring a quick delivery of stored nutrients as needed. The functions carried out by the subcutaneous fatty tissue, besides the storage of nutrients in the form of liquid fats, include the insulation of body from cold and shock absorption. Fats, also triglycerides or acylglycerins, are the most plentiful and simplest fatty acid-containing lipids. They are esters of triol alcohol, glycerine with three saturated and/or unsaturated fatty acids. Fats make up the main component the fat depots.

2.2 Porcine skin [20]

Porcine skin is similar to human skin in terms of color, hair follicles, sweat glands, and subcutaneous fat. The skin color for white pigs is convenient for identification of erythema. The presence of hair follicles is important, since skin stem cells (basal cells) lining the hair follicle bulb is thought to aid in the repopulation of the skin after irradiation. The skin of porcine is much thicker than that of humans. and that pig hide can be tanned. The thickness of epidermis varies from 70-140 μm, thus being within a range similar to that of human, i.e. 50-120 μm. Porcine epidermis varies in thickness depending on various body parts, mainly because of differences in the thickness of stratum corneum. The dermal-epidermal ratio of porcine skin varies from 10:1 to 13:1 and these measurements are comparable to those of human skin. The dermis of pigs is divided into 2 layers: the upper papillary layer and the lower reticular layer as shown in Figure 2.2. The papillary layer consists of numerous conical vascular eminences (the papillae) rising perpendicularly from the surface of dermis, while the reticular layer beneath this is the major part of dermis, consisting of strong interlacing bands of fibrous tissue. The thickness of dermis in the young (14-15

wk-old) pigs is approximately 2 mm. Networks of elastic fibers are found throughout the dermis and appear to be densest on dorsal aspect. The density of basal cells, cell kinetics, collagen fibers, and vascular supply are all similar to that of humans. Porcine skin is more human like than any other available animals.

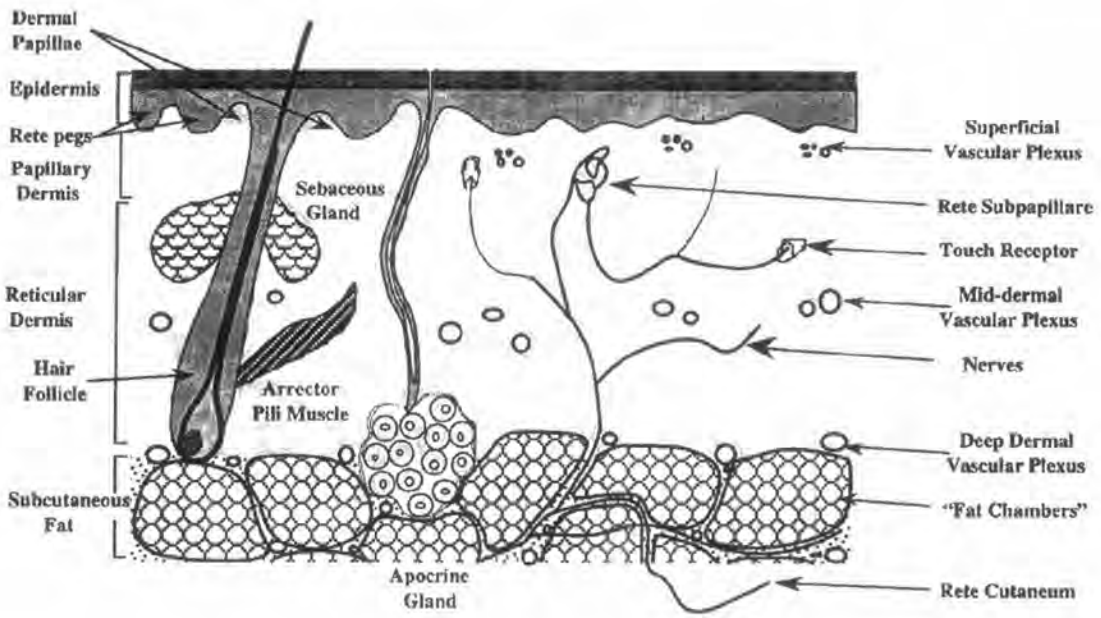

Figure 2.2 Anatomy of porcine skin [20]

2.3 Wound [21]

2.3.1 Type of wound

To facilitate proper treatment, wounds are classified as first, second, third and sometimes fourth degree as shown in Figure 2.3, depending on how deep a damage to skin tissue layers. The size of body area burned also helps classify a wound as a minor or major injury. A severe burn may also involve initial as well as delayed injury.

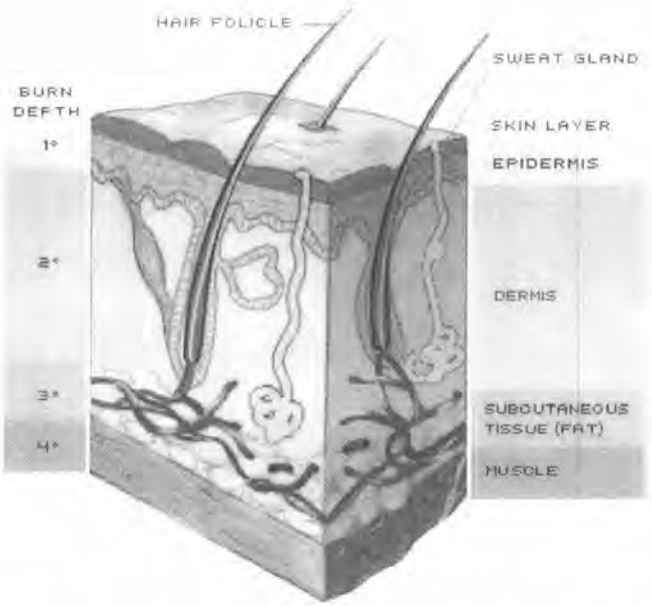

Figure 2.3 Wound depth diagram [21]

2.3.1.1 First degree wound

A first degree wound is limited to the epidermis. It is characterized by heat, pain, moistening and reddening of wounded surface, but rarely shows blistering or charring of tissue. First degree wounds often heal in three to seven days and rarely result in scar formation. Typical first degree wounds include sunburn and minor scalds.

2.3.1.2 Second degree wound

Sometimes referred to as partial thickness wounds, second degree wounds are characterized as either "superficial" or "deep." Both types penetrate deeper than a first degree wound and destroy epidermal layers, extending into dermis layer. They can cause damage to sweat glands and hair follicles and are extremely painful, often with intense swelling.

Skin that has incurred a superficial second degree wound is moist, red and weepy. Most superficial second degree wounds heal in 10 to 21 days, but leave a change in skin color and pigmentation. A deep second degree wound can be ivory or pearly white in color and may require a process known as debridement and additional skin grafting treatments. Deep second degree wound has completely destroyed epidermis and extend further into the dermis, with large amounts of necrotic tissue being presented. Both fluid and bacterial barriers are severely compromised, putting a patient at a much higher risk. These wounds, if allowed to heal on their own (because dermis has been grossly distorted or destroyed), result in hypertrophic scarring, with nonoptimal cosmetic results. These types of wound should be treated as if they were third degree wounds to allow faster and better healing.

2.3.1.3 Third Degree wound

A third degree wound, also known as a full thickness wound, destroys all the epidermal and dermal skin layers. The tissue damage extends below hair follicles and sweat glands to subcutaneous (fat) tissue. With this degree of wound, skin becomes charred and leathery and often appears depressed relative to surrounding tissue. Skin can be bright red, waxy white, tan or brown. There are no blisters and third degree wounds may cause massive swelling. Third degree wounds are not regenerated because the injury has destroyed nerve endings. Skin grafting or other replacement options are required for treatment of a third degree wound. When a burn injury is deep enough to involve muscle, bone, tendon and/or ligament, it is sometimes classified as a fourth degree wound. These burns are often life threatening and may require amputation. In case of third degree wounds, the wound is freely permeable to fluids, proteins and bacteria. The constant protienaceous exudates from these wounds, combined with an abundance of necrotic skin above, make an ideal media for bacterial growth. In these injuries, all epidermal cells with in the wound have been destroyed, including those in epidermal appendages. Without skin replacement, epidermal cells must migrate from wound edges, therefore, prompt debridement of wounds followed either by grafting or other methods of wound coverage is the treatment of choice. Small third degree wounds (those of less than 2 cm) may be

allowed to heal spontaneously by ingrowth from wound edges, but this process requires at least 6 weeks. The wound may never heal completely, always resulting in a generous scar.

2.3.2 Wound healing process [22]

An immediate response to skin injury is bleeding and the formation of a blood clot. Initially the vasculature constricts to decrease the blood flow but later it dilates and becomes more permeable allowing the blood plasma and various cell types to easily enter wound sites. During this 'inflammatory phase' of healing, many white blood cells aggregate in wounds, and these form an important source of regulatory chemicals in the later phases of repair. The upper part of blood clot dries out to form scab, and the epidermal layer of skin heals by direct cell migration underneath the scab. The lower part of blood clot provides a scaffold along which fibroblasts and other cells migrate into wounds, from surrounding and underlying tissue, and thus changes from an acellular region to a region rich in cells and capillaries known as granulation tissue. Within the wounds, fibroblasts can become actively contractile, and pull wound edges together. This causes tension lines familiar around wounds. The fibroblasts also break down blood clot and replace it with collagen based scar tissue. These healing processes in the dermal layer of skin depend on a dense network of capillaries, which form in response to injury, and are responsible for the red appearance of healing wounds.

The repair process described above can be conveniently divided into epidermal healing, changes in dermal matrix, and wound contraction.

2.3.2.1 Epidermal healing

Repair of the epidermis is usually fast and effective which occurs in healing of both deep and epidermis damaged wounds. Epidermal healing is a combination of two key processes: cell migration into wounds, and regulated cell division near wound edges. Both processes are regulated by a wide range of growth factors. For many

years, key growth factors regulating epidermal repair were thought to be members of the epidermal growth factor family. But in the mid-1990s, the work of Werner and coworkers showed that keratinocyte growth factor (KGF) plays a key role in epidermal healing, and is expressed at very high levels within 24 hours of injury. Although its effects are on epidermal keratinocytes, KGF is produced only within the dermal layer of skin. This implies that the regeneration of epidermis cannot be occurred without dermal support.

2.3.1.2 Repair of dermal extracellular matrix

The starting point for scar tissue formation is the blood clot, a dense random network of fibers made from protein fibrin. As fibroblast cells migrate into wounds from surrounding tissue, they break down fibrin, replacing it with a provisional extracellular matrix (ECM), which in turn is converted to a new dermal tissue whose key constituent is collagen. The fibroblasts will continue to reorganize collagen–based ECM for many months after wounding. Typically new dermal tissue is different from original dermal tissue and it is called a scar.

Two key features of scar tissue ECM that attracted the attention of empirical modeling are the details of collagen composition, and the orientation of fibers. Among different types of collagen, types I and III are most abundant in dermis, and the proportion of type III collagen is higher in scar tissue than in normal dermis. Since type III collagen decorates the surface of type I fibrils, this results in thinner collagen fibers. The balance between types I and III collagen is regulated by the different isoforms of transforming growth factor β (TGF-β). Although collagen type is different in scar tissue and normal dermis, differences in fiber orientation are more significant. In normal dermis, collagen fibers are arranged in a random, 'basket-weave' form, whereas in a scar, there is a predominant direction of alignment. The difference in fiber alignment causes a scar to look different from surrounding skin, and it also gives scar poorer mechanical properties. The presence of predominant fiber orientations means that scar tissue is anisotropic. A number of inorganic materials, such as graphite, are anisotropic, and there are established methods for studying such

materials mathematically. But scar tissue is much more complicated because the anisotropy varies dynamically in space and time, as fibroblasts move into wounds and fibrin is replaced by collagen.

Conventionally, if dermis is removed, the wound contracts and a scar forms. Scar tissue, which is less flexible than physiologically normal dermis, can lead to restricted motion at a joint. If, instead, a synthetic matrix is used, contraction is inhibited and there is little scar formation. The formation of scar tissue is associated with wound contraction.

2.3.1.3 Wound contraction

The familiar tautness of skin around wounds is evidence of wound contraction, the process by which contractile fibroblasts within wounds pull their edges together. This occurs to a much greater extent in animal skin than in humans, because of the absence of *panniculus carnosus*, a muscle that connects human dermis to underlying tissue.

The contractile fibroblasts within wounds are thought by some investigators to have a separate phenotype, while others regard the traction force as parts of normal fibroblast behavior. But the basic phenomenon is universally agreed. Wound contraction occurs by the following sequence of events. In normal dermis, skin fibroblasts are inactive. After wounding, fibroblasts proliferate and synthesize a new collagen matrix called granulation tissue. Migrating fibroblasts at the edges of the wound initiate contraction. As contraction continues, the resistance of wounds to further contraction increases and the contracting fibroblasts at the edges of wounds differentiate into myofibroblasts. Myofibroblasts are elongated cells with actin filament bundles (also known as stress fibers) oriented along the lines of greatest resistance, allowing them to further contract the wounds. Differentiation of fibroblasts to myofibroblasts has been shown to depend at least on the extent to which the wound resist contraction. After the wounds have healed, fibroblast population decreases and extracellular matrix remodelling begins.

Wound contraction and wound modelling is the last step in wound healing. Due to wound contraction, healed wounds gain strength. However the maximal strength of scar is only approximately 70% of normal skin.

2.3.1.4 Wound angiogenesis

The macrophages that enter wounds as parts of the initial inflammatory phase of healing produce a range of growth factors. Some of these regulate fibroblast influx, such as TGF-β and attract endothelial cells into wound space, leading to the establishment of a capillary network, which can constitute as much as half of wounds. This process of capillary ingrowth, known as angiogenesis, is essential to healing, since the high levels of metabolic activity in a healing wound cannot occur without a specific blood supply. In fact, from a clinical viewpoint, one of the most pressing concerns in wound healing is the failure of some wounds to heal, because of an inadequate vascular response. The biology of angiogenesis has been well studied, mainly in the context of cancer rather than wound repair. In response to chemical gradients, the endothelial cells which line blood vessels near wounds begin to migrate, forming small, finger-like capillaries. Cells proliferation within these sprouts causes the capillaries to extend into wounds and eventually merge (anastomosis), leading to a capillary network.

2.4 Cell-extracellular matrix interaction [24]

The goal of tissue engineering is to regenerate living tissue substitutes in order to replace or enhance lost tissue function or structure. To accomplish this, tissue cells must be organized and behave as if they are parts of original tissue in vivo. In other words, cells must believe that they are in their native environment, and receive all signals from environment allowing cell adhesion, cell migration, differentiation and proliferation. In most cases, a critical component of these processes is the way in which cells interact with the surrounding extracellular matrix (ECM).

Extracellular matrix (ECM) is a structural material that lies beneath epithelia and surrounds connective tissue cells. ECM acts as a support material created by cells as scaffolding on which to reside. However, a cell can interact with its own ECM products as well as ECM produced by other cells via specific cell surface receptors, thereby linking ECM to cell interior via intracellular signaling processes. These cell surface receptors can respond to cell signaling events as well as transduce specific cell signals. In this way, various cell functions are continuously related to, and dependent on the composition and organization of matrix. In vivo, the interaction between a cell and ECM can affect cell and tissue differentiation, cell migration, cell proliferation, cell adhesion and tissue regeneration and repair. Local variations in the composition or organization of ECM can therefore give rise to spatial and temporal variations in tissue structure and function. In developing organisms, ECM is constantly being degraded, remodeled and resynthesized locally, changing the environment in which tissue cell resides.

2.4.1 Composition of ECM [5]

ECM is a complex mixture of structural and functional proteins, glycosaminoglycans, glycoproteins and small molecules arranged in a unique, tissue specific three-dimensional architecture. The logical division of ECM into structural and functional components is not possible because many of these molecules have both structural and functional roles in health and disease.

2.4.1.1 Collagen

Collagen is the most abundant protein within mammalian ECM. Greater than 90% of the dry weight of ECM from most tissues and organs is represented by collagen. More than 20 distinct types of collagen have been identified, each with a unique biologic function. Type I collagen is the major structural protein presented in tissues. Type I collagen is abundant in tendinous and ligamentous structures and provides necessary strength to accommodate uniaxial and multiaxial mechanical loading to which these tissues are commonly subjected. These tissues provide a

convenient source of collagen for many medical device applications. Bovine Type I collagen is harvested from Achilles tendon and is perhaps the most commonly used xenogeneic ECM component intended for therapeutic applications.

2.4.1.2 Fibronectin

Fibronectin is second only to collagen in quantity within the ECM. Fibronectin is a dimeric molecule of 250,000 MW subunits. It exists both in soluble and tissue isoforms and possesses ligands for adhesion of many cell types. The ECM of submucosal structures, basement membranes and interstitial tissues all contain abundant fibronectin. The cell friendly characteristics of this protein have made it an attractive substrate for in vitro cell culture and for use as a coating for synthetic scaffold materials to promote host biocompatibility. Fibronectin is rich in Arg-Gly-Asp (RGD) subunit; a tripeptide that is important in cell adhesion via the $\alpha_5\beta_1$ integrin. Fibronectin is found at an early stage within the ECM of developing embryos. It is important for normal biologic development, especially the development of vascular structures.

2.4.1.3 Laminin

Laminin is a complex adhesion protein found in the ECM, especially within basement membrane. This protein plays an important role in early embryonic development. This trimeric cross-linked polypeptide exists in numerous forms dependent upon the particular mixture of peptide chains (e.g. $\alpha 1$, $\beta 1$, $\gamma 1$). The prominent role of laminin in the formation and maintenance of vascular structures is particularly noteworthy when considering the ECM as a scaffold for tissue reconstruction. The crucial role of the beta-1 integrin chain in mediating hematopoietic stem cell interactions with fibronectin and laminin has been firmly established. Loss of the beta-1 integrin receptors in mice results in intrapartum mortality. This protein appears to be among the first and most critical ECM factors in the process of cell and tissue differentiation. The specific role of laminin in tissue reconstruction when ECM is used as a scaffold for tissue and organ reconstruction in

adults is unclear but its importance in developmental biology suggests that this molecule is critical for organized tissue development as opposed to scar tissue formation.

2.5 Skin substitute [23,25]

Major injuries resulting in extensive damage to the skin necessitate immediate coverage to aid repair and regeneration of skin. However skin injuries are traumatic events, which are seldom accompanied by complete structural and functional restoration of original tissues. There is a substantial expense by way of health care and loss of personal freedom, quality of life and productivity. Research efforts to deal with skin loss, have focused on three principal directions. Firstly, towards improvement of wound healing by factors which speed up the process and reduce scarring. Secondly, the design and development of skin substitutes as functional equivalents of autograft skin. Thirdly, to identify cues that induce skin to heal by regeneration rather than repair (scarring). Current treatment modalities involve multiple stages. Excision of injured tissue as the first step, subsequent to stabilizing the patient in the case of a burn wound, represents a major advance in treatment. This is followed by grafting which involves application of skin substitutes which are either split thickness autografts, allografts (homografts), xenografts (heterografts).

Research and development in the field of wound dressing has resulted in the fabrication and production of a wide variety of synthetic and biological dressings. Synthetic dressings based on polymeric sheets are available as Tegaderm, Dermafilm, Opsite, Praflex, etc. These are only suitable for superficial wounds and not for larger wounds. Biological dressings include allograft, xenograft from pigs and amniotic membrane, reconstituted collagen films and sponges from bovine and other sources. Allografts from cadavers carry a risk of viral contaminants and are potentially antigenic, hence they will be rejected if immunosupressents are not administered. Owing to the disadvantages of allografts, acellular dermis was developed. Acellular dermis can be treated with chemical agents to remove cellular components, which contribute to antigenicity leaving an immunological inert acellular dermal matrix and

intact basement membrane complex. This type of acellular dermal transplant (AlloDerm) was developed and marketed by Life Cell Corporation, Woodlands, Texas, to act as a template and to regenerate into a viable dermis when grafted into a full thickness burn. Use of Allo Derm began in 1992 for burn patients. Since all the cells are removed, no component necessary for survival and transmission of viruses is presented.

Acellular dermis, which provides a dermal component in deep burns, requires the supply of cadaver skin from established skin banks. In many countries, there is no provision of skin banks for cultural and ethical reasons. Hence an alternative dermal component has to be fabricated. The understanding of the complexity of essential components in dermis has enabled the production of a composite graft made up of a bi-laminated membrane consisting of a silicone outer covering bonded to a collagen based dermal analog (Bovine collagen and chondroitin sulfate) by Burke et al., named as Integra. Neo-vascularization of this dermal substitute has been observed, but the resultant tissue resembles granulation tissue rather than normal dermis and reconstituted collagen matrices do not posses exact three dimensional structures. Integra artificial skin is indicated for the postexcisional treatment of life threatening full thickness or deep partial thickness thermal injury where sufficient autograft is not available at the time of excision or not desirable due to the physiological condition of patients.

2.6 Acellular dermis [6]

2.6.1 History and roles of acellular dermis

Acellular dermis has been used since the mid-1970s to augment damaged soft tissue structures. Most early studies retained epidermal layer in the graft and utilized skin derived from pigs. More recently, however, allogenic tissue has become a preferred tissue source in order to avoid the risk of virus transfer across species and to decrease the incidence of immunologic rejection. In the early 1900's, cryopreserved cadaver skin was used to investigate the utility of using acellular dermis as a growth

substance for cells. Autologous keratinocytes were seeded on the acellular dermis as a mean of forming a "reconstituted skin" that was then engrafted into athymic mice. Results showed that engraftment of the reconstituted skin occurred within 2 weeks and that the formally acellular dermis was repopulated by fibroblast and blood vessels of host origin. While acellular dermis is still used in vitro to form cell-tissue implant, implantation of acellular dermis without added cells has become favored approach for many clinical applications.

Allogenic acellular dermis was first used clinically in 1995 to manage full-thickness wound. Day 16 biopsies of the grafted acellular dermis revealed that keratinocytes had spread though the interstices of the meshed graft and formed a complete covering on acellular dermis. Host cell infiltration and neovascularization of the grafted area underlying dermal matrix was observed. There was minimal inflammation and no host specific immune response. When evaluated using electron microscopy, the same biopsy revealed a layer of organized collagen and extensive fibroblast infiltration into processed dermal matrix. Persistence of elastin was also evident. The skin healed with minimal scar information in comparison to an untreated site on the same patient. This demonstrated that acellular dermis supported fibroblast infiltration, neovascularization, and epithelialization in an absence of an inflammatory response.

The favorable reports of reduced scarring, rapid graft incorporation, and angiogenesis, led to the use of acellular dermis in several other tissue repair applications. Acellular dermis has been used for dural replacement, internal soft tissue repair, and small diameter vascular replacement. However, the primary use of acellular dermis remains in surgical reconstruction of skin following full and partial thickness skin wound and burns.

While acellular dermis has been successful as a dermal substitute and offers promising uses for future applications, its use is not without complications and drawbacks. The risk of using allogenic source tissue, in term of viral and disease transfer, cannot be completely avoided even though transmission risk has virtually

declined to zero with the advent of rigorous screening and safety testing. In addition, the only published study investigating acellular dermis as a vascular graft revealed questions regarding long-term permanence, which was also a major concern of using acellular dermis for reconstructive plastic surgery. Patency rates of acellular dermis after 28 days as a 4-mm diameter vascular graft were at 90%. Of great concern, however, was the finding that 60% of these initial grafts formed false aneurysms and only 30% of the patent grafts had endothelial luminal linings after 28 days. In burn grafting, permanence is less of an tissue than is temporary barrier function and prevention of inflection. Issues regarding prolonged effect require further study if acellular dermis is to be used in locations where long-term strength is needed, but the use of acellular dermis as a dermal graft that leads to neovascularization and subsequent engraftment appears secure.

2.6.2 Characteristics of acellular dermis

Acellular dermis is harvested from either porcine skin or human cadaver skin. The marix derived from full or split-thickness skin treated to remove epithelial components (keratinocytes, sweat glands and subcutaneous fat) and dermal components (fibroblasts, vascular endothelium and smooth muscle). It is comprised of normal dermal tissue structure that remains after the cells are removed. Like other naturally occurring biopolymers, acellular dermis is rich in collagen type I. High level of the type IV and type VII collagen composition of native dermis are also retained. In addition to collagen, the elastin content of the dermis is also retained during processing, leading to a graft construct with favorable elastic properties. Additional component structures of the acellular dermis have not yet been identified. These dermal substitutes must exhibit three important properties; very low antigenicity, the capacity for rapid vascularization, and stability as a dermal template.

2.7 Decellularized process of tissue and organ [8]

The most robust and effective decellularization protocols include a combination of physical, chemical, and enzymatic approaches. A decellularization protocol generally begins with lysis of the cell membrane using physical treatments or ionic solutions. Separation of cellular components from the ECM was carried out by using enzymatic treatments, then follow by detergents in order to solubilize of cytoplasmic and nuclear cellular component. These steps can be coupled with mechanical agitation to increase their effectiveness. Following decellularization, all residual chemicals must be removed to avoid an adverse host tissue response to chemicals. The efficiency of decellularization and preservation of ECM can be assessed by several methods such as enzymatic and chemical treatments. The mechanisms of physical, enzymatic, and chemical decellularization for a variety of tissues are reviewed in the following sections.

2.7.1 Physical method

Physical methods that can be used to facilitate decellularization of tissues include freezing, direct pressure, sonication, and agitation. Snap freezing has been used frequently for decellularization of tendinous and ligamentous tissue and nerve tissue. By rapidly freezing a tissue, intracellular ice crystals form that disrupt cellular membranes and cause cell lysis. The rate of temperature change must be carefully controlled to prevent ice formation from disrupting ECM as well. While freezing can be an effective method of cell lysis, it must be followed by processes to remove cellular materials from tissues. Cells can be lysed by applying direct pressure to tissue, but this method is only effective for tissues or organs that are not characterized by densely organized ECM (e.g. liver, lung). Mechanical force has also been used to delaminate layers of tissue from organs that are characterized by natural planes of dissection such as small intestine and urinary bladder. These methods are effective, and cause minimal disruption to the three dimensional architecture of ECM within these tissues. Mechanical agitation and sonication have been utilized simultaneously with chemical treatment to assist in cell lysis and removal of cellular debris.

Mechanical agitation can be applied by using a magnetic stir plate, an orbital shaker, or a low profile roller. There have been no studies performed to determine the optimal magnitude or frequency of sonication for disruption of cells, but a standard ultrasonic cleaner appears to be as effective at removing cellular material as placing tissue on an orbital shaker. In all of these procedures, the optimal speed, volume of reagent, and length of mechanical agitation is dependent on the composition, volume, and density of tissues.

2.7.2 Chemical methods

2.7.2.1 Alkaline and acid treatments

Alkaline and acid treatments are used in decellularization protocols to solubilize the cytoplasmic component of cells as well as remove nucleic acids such as RNA and DNA. For example, acetic acid, peracetic acid (PAA), hydrochloric acid, sulfuric acid, and sodium hydroxide (NaOH) can effectively disrupt cell membranes and intracellular organelles. However, these chemicals also dissociate important molecules such as GAGs from collagenous tissues. The effects of PAA treatment on ECM components have been studied extensively. Several types of collagen including types I, III, IV, V, VI, and VII have been identified following treatment with PAA, however, the microstructure of collagen fibers has not been closely examined following such treatment. The ECM retains many of native GAGs including hyaluronic acid, heparin, heparin sulfate, chondroitin sulfate A, and dermatan sulfate following PAA treatment. It has also been shown that laminin and fibronectin are presented in ECM scaffolds following exposure to PAA. Decellularization and disinfection with PAA have been successfully used for many tissue-engineering applications.

2.7.2.2 Non- ionic detergents

Non-ionic detergents have been used extensively in decellularization protocols because of their relatively mild effects upon tissue structure. Non-ionic detergents

disrupt lipid–lipid and lipid–protein interactions, but leave protein–protein interactions intact so that proteins within a tissue or organ following non-ionic detergent treatment should be left in a functional conformation. Triton X-100 is the most widely studied non-ionic detergent for decellularization protocols. When Triton X-100 was used to decellularize a heart valve, complete removal of nuclear material was observed with maintenance of valvular structure after 24 h. However, cellular material was found in the adjacent myocardium and aortic wall. With regard to the ECM components, Triton X-100 led to a nearly complete loss of GAGs and decreases in the laminin and fibronectin content of the valve tissue. Although Triton X-100 can be an effective decellularization method, its efficacy is dependent upon tissues being decellularized and the other methods with which it is combined in a given decellularization protocol.

2.7.2.3 Ionic detergents

Ionic detergents are effective for solubilizing both cytoplasmic and nuclear cellular membranes, but tend to denature proteins by disrupting protein–protein interactions. The most commonly used ionic detergents are sodium dodecyl sulfate (SDS), sodium deoxycholate and Triton X-200. SDS is very effective for removal of cellular components from tissues. Compared to other detergents, SDS yields more complete removal of nuclear remnants and cytoplasmic proteins, such as vimentin SDS tends to disrupt the native tissue structure, and causes a decrease in the GAG concentration and a loss of collagen integrity. However, it does not appear that SDS removes collagen from tissues.

2.7.3 Enzymatic methods

Enzymatic techniques of decellularization include the use of protease digestion, calcium chelating agents, and nucleases. Trypsin is one of the most commonly used proteolytic enzymes in decellularization protocols. Trypsin is a highly specific enzyme that cleaves the peptide bonds on the carbon side of arginine and lysine if the next residue is not proline. The maximal enzymatic activity of trypsin

occurs at 37°C and at a pH of 8. Nucleases such as endonucleases catalyze the hydrolysis of the interior bonds of the ribonucleotide or deoxyribonucleotide chains whereas exonucleases catalyze the hydrolysis of the terminal bonds of deoxyribonucleotide or ribonucleotide ultimately leading to the degradation of RNA or DNA. The efficacy of enzymatic treatments for removal or separation of cellular materials from ECM has been studied for a variety of tissues. Enzymatic methods of decellularization have an adverse effect on the extracellular components of tissues and organs. Prolonged treatment with trypsin causes disruption of the collagen in the tissue. Trypsin/EDTA does, however, substantially reduce the laminin and fibronectin content of ECM. Prolonged exposure to trypsin/EDTA greatly decreases the elastin content and GAGs (chondroitin sulfates, keratin sulfates, and dermatan sulfates) over time. Such treatments can contribute to a decrease in tensile strength of up to 50%. The remaining ECM after such enzymatic decellularization protocols still supports endothelial cell growth in vitro despite the removal of ECM components. It is desirable to limit the duration of exposure to trypsin/EDTA treatments to minimize the disruptive effects upon the ultrastructure and composition of ECM.

2.8 Cell-dissociating enzymes [26]

Enzymes that have been satisfactory used in cell isolation for a wide variety of tissues from various species and ages are collagenase, trypsin, and dispase.

Collagenase

Bacterial collagenase is a crude complex containing a collagenase more accurately referred to as clostridiopeptidase which is a protease with a specificity for the X-Gly bond in the sequence Pro-X-Gly-Pro, where X is most frequently a neutral amino acid. Such sequences are often found in collagen, but only rarely in other proteins. While many proteases can hydrolyze single-stranded, denatured collagen polypeptides, clostridiopeptidase is unique among proteases in its ability to attack and degrade the triple-helical native collagen fibrils commonly found in connective tissue.

True collagenase may cleave simultaneously across all three chains or attack at a single strand. Mammalian collagenases split collagen in its native triple-helical conformation at a specific site yielding fragments, TC A and TC B, representing 3/4 and 1/4 lengths of the tropocollagen molecule, respectively. After fragmentation the pieces tend to uncoil into random polypeptides and are more susceptable to attack by other proteases.

Purified clostridiopeptidase alone is usually inefficient in dissociating tissues due to incomplete hydrolysis of all collagenous polypeptides and its limited activity against the high concentrations of non-collagen proteins and other macromolecules found in ECM. The collagenase most commonly used for tissue dissociation is a crude preparation containing clostridiopeptidase A in addition to a number of other proteases, polysaccharidases and lipases. Crude collagenase is well suited for tissue dissociation since it contains the enzyme required to attack native collagen and reticular fibers in addition to the enzymes which hydrolyze the other proteins, polysaccharides and lipids in ECM of connective and epithelial tissues.

Trypsin

Trypsin is a pancreatic serine protease with specificity for peptide bonds involving the carboxyl group of the basic amino acids, arginine and lysine as shown in Figure 2.4. Trypsin is one of the most highly specific proteases known, although it also exhibits some esterase and amidase activity. Trypsin consists of a single chain polypeptide of 233 amino acid residues. It is produced by removal of N-terminal hexapeptide from trypsinogen which is cleaved at the Lys^{6}-Ile^{7} peptide bond. The amino acid sequence of trypsin is crosslinked by 6 disulfide bridges. The active site amino acid residues of trypsin include His^{46} and Ser^{183} [1,3]. Trypsin has the optimum activity at 37°C and pH 7-9.

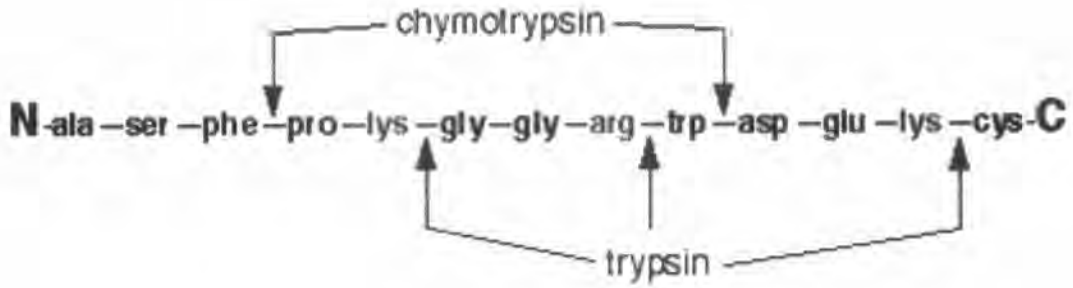

Figure 2.4 Peptide bond cleavage specificity of trypsin

Purified trypsin alone is usually ineffective for cells dissociation since it shows little selectivity for extracellular proteins. Combinations of purified trypsin and other enzymes such as elastase and/or collagenase have proven effective for dissociation. In tissue culture laboratories, researchers use purified trypsin to release cells into suspension from monolayers growing on the interior surfaces of culture vessels. Most cells originating from normal tissues and not highly adapted to artificial culture conditions grow in monolayers. Because such cells are more like cells in normal tissues, many tissue culture researchers are studying cells that grow in monolayer culture. Monolayer cultures are commonly grown in glass or polystyrene roller bottles, culture flasks, or petri dishes. Plastic vessels used in tissue culture work are specially treated to ensure good adherence of cells to vessel walls.

Neutral protease (Dispase)

Neutral protease (Dispase) is a bacterial enzyme produced by *Bacillus polymyxa* that hydrolyses N-terminal peptide bonds of non-polar amino acid residues. It is classified as an amino-endopeptidase. Dispase can be divided into two types: dispase I and dispase II. Dispase I has approximately 10-fold higher specific activity than dispse II. Dispase cleaves fibronectin, collagen IV, and to a lesser extent collagen I, but it does not cleave collagen V. Its mild proteolytic action makes the enzyme especially useful for the isolation of primary and secondary (subcultivation) cells since it maintains cell membrane integrity. The optimum temperature and pH of dispase activity are 37°C and 7-9, respectively.

Dispase is also frequently used as a secondary enzyme in conjunction with collagenase and/or other proteases in many primary cell isolation and tissue dissociation applications. Dispase dissociates fibroblast-like cells more efficiently than epithelial-like cells so it has also been used for differential isolation and culture applications. Other advantages are its non-mammalian (bacterial) source and its ability to be inhibited by EDTA. Dispase yields a single cell suspension far more gently and effectively than trypsin, collagenase or other proteolytic enzymes. It will not harm cells harvested for subcultivation or bioassays.