CHAPTER II
LITERATURE REVIEW

A. Collagen structure and type

Tissues are not exclusively formed by cells. They also have substantial part called 'extracellular space' for complicated network of macromolecules constitution. The substance filled in this space is composed of various protein and polysaccharides, generally secreted from localized cell and then organized into network. These substances are extracellular matrix. Among extracellular matrices, collagen is the largest fibrous proteins family found in multicellular animals. The structural protein is secreted by various types of connective tissue cell like fibroblast and form a supramolecular assembly in the extracellular matrix further. It is a major component of skin, bone and tendon (Albert et al., 2002). Approximately 30% of proteins in animals are collagens. Bone and tendon consist of more than 90% collagen and more than 50% of extracelluar matrix in skin is skin (Friess, 1998).

Each collagens has a unique structure, size and amino acid sequence. The primary feature of its molecule is its long, stiff and triple stranded helical structure (Albert et al., 2002) which consists of three alpha chains twined around one another in a ropelike superhelix (Lee et al., 2001). In order to synthesize the alpha chain that formed the collagen triple helices, the repeating tripeptide sequences like [Gly (glycine)-X-Y]$_n$ are required. X and Y can be any amino acid but proline (Pro) is mostly found in the X position and 4-hydroxyproline (Hyp) is predominantly in the Y position. Hyp is derived from post-translational hydroxylation of proline mediated by prolylhydroxylase (Friess, 1998). Additionally, every third amino acid, situated in the centre of the triple helices in a very restricted space can only be Gly. Therefore, the collagen molecules are extremely enriched of Pro, Hyp and Gly that have an effect on formation of their helical structures. Pro can stabilize their conformation by its ring structure while Hyp is essential for forming interchain hydrogen bond. Gly- the smallest amino acid also has an impact on three helical alpha chains by packing them tightly together in order to form the final collagen superhelix (Albert et al., 2002). Besides these amino acids, collagen also contains an unusual amino acid hydroxylysine which is formed similar to the hydroxyproline. Hydroxylysine is synthesized from lysine in the endoplasmic reticulum via enzymatic hydroxylation of

lysyl hydroxylase. This amino acid can stabilize the triple helix by forming the interchain hydrogen bond as a result in limiting rotation of molecule and stiffening of alpha helices (Friess, 1998). Thus, mutation of collagen by more bulky amino acid residue substitutes Gly in the triple helical domain causes severe disruption of collagen structure. This evidence leads to connective tissue abnormalities like osteogenesis impecfecta (Yamauchi, 2002).

Three-stranded polypeptide chain is called tropocollagen. The structure has a pitch approximately 8.6 nm, length 300 nm with a diameter of 1.5 nm and averaged molecular weight (MW) respecting 300 kDa. This extreme ratio of the dimensions results in high viscosity in solutions and high mobility in electrical fields. Besides the helical region, the collagen has 9-26 amino acids at amino terminal (N-terminal) and carboxyl terminal (C-terminal) chain ends of the molecule which are not incorporated into the triple helical structure. These non-helical polypeptides are called N- and C- telopeptides respectively. (Figure 2.1)

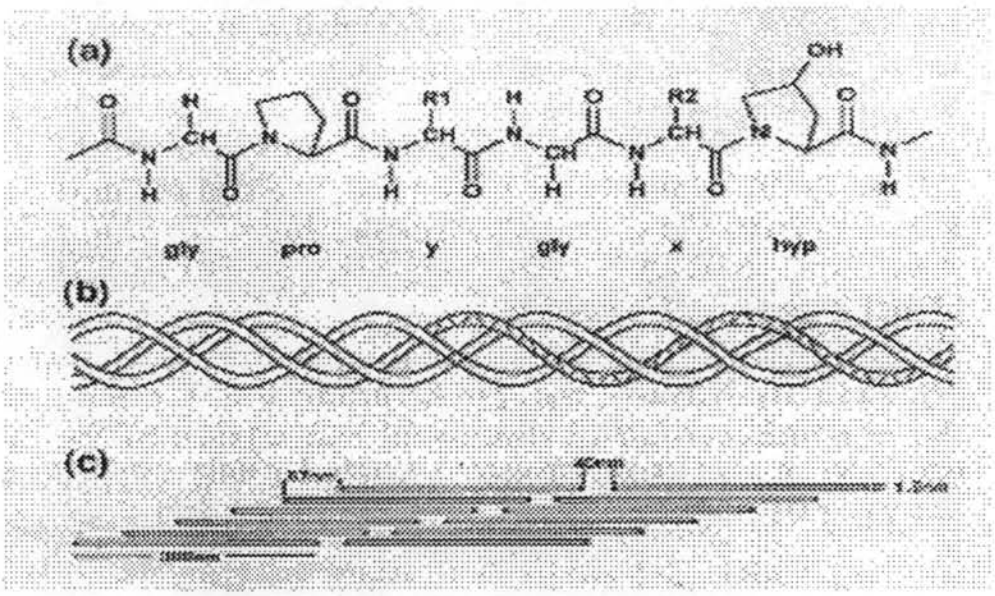

Figure 2.1 Chemical structure of collagen type I (a) primary amino acid sequence, (b) secondary triple helix structure and (c) Staggered quaternary (Friess, 1998)

The quaternary structure of the collagen molecule is generated by fibrilogenesis mechanism aggregating the 4-8 collagen molecules into microfibril.

The fibrils reach from 10-500 nm in diameters depending on tissue type and developmental stage. The arrangement of helical chains is staggered by 67 nm with an additional gap of 40 nm between succeeding molecules.(Figure 2.1) After that the collagen fibrils are organized into fibers and some parts of which can form larger fiber bundles (Friess,1998).

Thus the resilience of the fibers is the result of systematic packaging of triple helices. In addition, it also derives form intra- and intermolecular crosslinks. During fibril formation, the lysyl oxidase influences crosslink formation. This enzymatic activity has an impact on only the non-helical telopeptide regions and the activity leads to conversion of selective lysine and hydroxylysine residues to the corresponding aldehydes allysine and hydroxyallysine. While the fibril is associating, the reaction of aldehydes can spontaneously occur. Therefore, the intramolecular crosslink between two α-chains of the same molecule at the non-helical part occurs by aldol condensation of two aldehydes. In case of intermolecular crosslinks between the telopeptides region of one collagen and helical region of an adjacent molecule is the result from aldimine formation (non-, mono- or dihydroxylated dehydrolysinonorleucine) (Δ-HLNL) between aldehyde residues and ε-amino groups presented by lysine and hydroxylysine. The inter-chain crosslinks are still reactive and continue to form polyfunctional crosslinks through multiple condensations with histidine, lysine or hydroxylysine residues (Friess,1998).

Because of its specific aggregation and crosslinking, collagen can form fiber which has unusual strength and stability. Consequently, collagen is the valuable extracellular matrix for the medical applications. However, the degree of crosslink can be increased with age (Bailey et al., 1998; Friess, 1998) and stress which influences to the properties of collagen materials (Friess, 1998).

According to Yamauchi (2002), collagen is encoded by a family of at least 38 distinct genes. In consequence, there are more than 21 different genetic types. Each collagen type shows marked diversity and complexity in the structure such as their length, assembly mode and number of amino acid in collagenous and non-collagenous portions as well as the extent of post-translational modifications. Furthermore, their quantities, biological functions and tissue distribution are unique. Therefore, the growing collagen superfamily can be classified into 3 major groups based on assembly mode and some structural feathers including fibril forming

collagens, Fibril Associated Collagen with interrupted Triple helices (FACIT) and non-fibrillar collagens as shown in Figure 2.2.

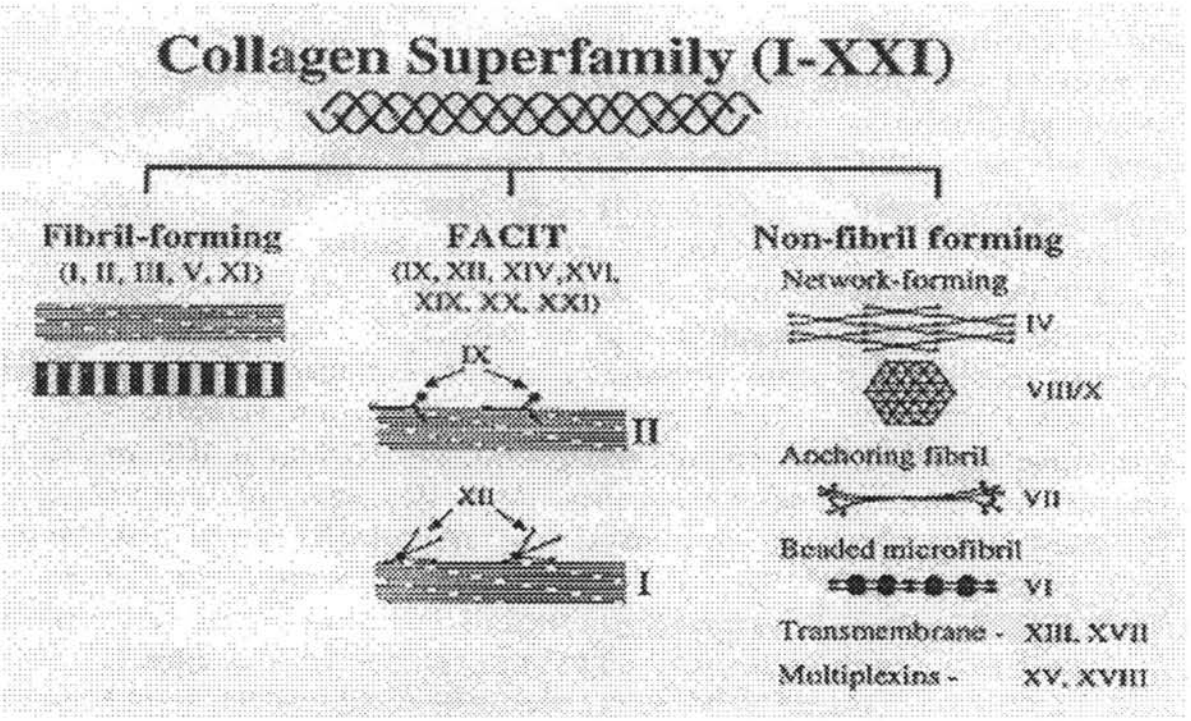

Figure 2.2. Collagen superfamily categorized by assembly modes and domain structure (Yamauchi, 2002)

The fibril forming collagen is the collagen group which is able to form fibrils in the extracellular matrix and then pack with neighboring collagen molecules into a quarter-staggered array. All of the fibril forming collagens consists of three domains which are composed of a short-amino terminal non-triple helical domain (N-telopeptide), a long central uninterrupted triple helical domain and short carboxy-terminal non-triple helical domain (C-telopeptide) (Figure 2.3). This group includes collagen type I, II, III, V and XI (Figure 2.2). It is general to find that collagen fibrils consist of collagen more than one types. Thus, these fibrils are called heterotypic collagen fibrils. For example, collagen type V can be found in the collagen type I fibril 5-10% and type XI are the minor component in the type II collagen. These minor fibrillar collagens (type V and XI) frequently retain at the amino propeptide (N-propeptide) because of incomplete extracellular processing. Whenever the collagen molecules are incorporated into fibrils, this portion will exist at the fibril surface. It

plays a role in regulating the lateral aspect of fibril growth by exerting steric hindrance in order to accretion of collagen molecule to the surface of the fibrils. The ratio of these minor fibrillar collagens such as type V and XI to the major ones like type I and II has been demonstrated that it may be one determinant of collagen fibril diameter.

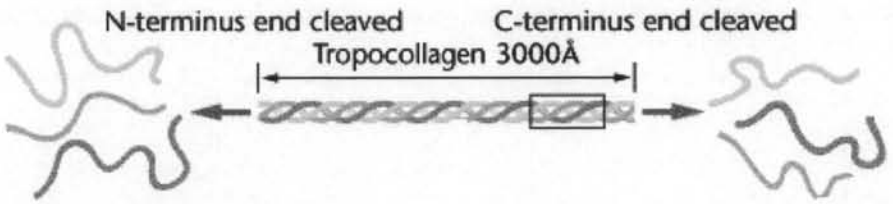

Figure 2.3. Fibril forming collagen structure with C-terminus and N-terminus (modified from Griffiths, 2000)

The second group is Fibril Associated Collagen with interrupted Triple helices (FACIT). The collagens in this group do not form fibrils themselves. They are necessary to attach to surface of the preexisting collagen fibrils such as type I and II. This group includes type IX, XII, XIV, XVI, XIX, XX, and XXI. These collagen fibrils consist of two or more short triple helical domains interrupted by short non-collagenous regions (Figure 2.2). They act as the bridging molecule between fibrils or between fibril and other matrix molecules or other cell because they have the projection of amino terminal globular domains extending from the fibril surface into the matrix.

The last group is non-fibrillar collagen. The character of the collagen is that they do not form fibril neither themselves nor closely structure. These collagens can be classified into many subgroups namely net work forming collagens, anchoring fibril collagens, microfibrillar collagen and multiplexin (Multiple triple helix domains with interruptions) as well as transmembrane collagens (Figure 2.2).

Type IV collagen is a representation of net work forming collagen subgroup. It is a major compartment of basement membrane. This type is important in separating cell or cell sheets from an underlying tissue. Moreover, it also plays a

role in cell infiltration, regulation of cell migration and growth as well as cell differentiation

Type VI collagen is represented in microfibrillar subgroup. The characters of microfibrillar subgroup are highly glycosylated, cysteine rich. Moreover, it is also composed of short triple helical domain and very large terminal non-collagenous globular domains accounting for more than two thirds of its molecule. Type VI collagen generally is a component of adhesive protein, therefore its function is associated with interaction between the α1 integrin family and other cells or extracellular matrix such as proteoglycan, fibrillar collagen and glycosaminoglycan. The lacking of the collagen expression has been suggested that it causes the metastasis of mesenchymal tumor cells.

The major member of anchoring fibrils is type VII collagen. The collagen can be found at the dermal-epidermal junction. It links the basement membrane to anchoring plaques in the underlying dermis. The mutation of this gene causes an abnormality called "Dystrophic epidermolysis bullosa''.

Multiplexin and transmembrane collagens are the last two subgroups. The type XV and XVII collagen are representatives of these subgroups respectively. Researchers have found that C-terminal fragment of both of the collagen types namely endostatin are a potent inhibitor of angiogenesis and tumor growth. Type XV collagen is found at basement membrane of capillary and skeletal muscle cell. Moreover, type XVII collagen has been demonstrated as a component of vascular and epithelial cell membrane.

Table 2.1. Chain composition and body distribution of collagen types, modified form (Friess, 1998)

Collagen type	Chain composition	Tissue distribution
I	$(\alpha1(I)_2\alpha2(I)$, trimer$(\alpha1(I)_3)$	Skin, tendon, bone, cornea, dentin, fibrocartilage, large vessels, intestine, uterus, dentin, dermis, tendon
II	$(\alpha1(II))_3$	Hyaline cartilage, vitreous, nucleus pulposus, notochord
III	$(\alpha1(III))_3$	Large vessels, uterine wall, dermis, intestine, heart valve, gingival (usually coexist with type I except in bone, tendon, cornea)
IV	$(\alpha1(IV)_2\alpha2(IV))$	Basement membranes
V	$\alpha1(V)\alpha2(V)\alpha3(V)$ or $\alpha1(V))_2\alpha2(V)$ or $(\alpha1(V))_3$	Cornea, placental membranes, bone, large vessels, hyaline cartilage, gingival
VI	$\alpha1(VI)\alpha2(VI)\alpha3(VI)$	Descemet's membrane, skin, nucleus pulposus, heart muscle
VII	$(\alpha1(VII))_3$	Skin, placenta, lung, cartilage, cornea
VIII	$\alpha1(VIII)\alpha2(VIII)$ chain organization of helix unknown	Produced by endothelial cells, Descemet's membrane
IX	$\alpha1(IX)\alpha2(IX)\alpha3(IX)$	Cartilage
X	$(\alpha1(X))_3$	Hypertrophic and mineralizing cartilage
XI	$1\alpha2\alpha3\alpha_1$ or $\alpha1(XI)\alpha2(XI)\alpha3(XI)$	Cartilage, intervertebral disc, vitreous humour
XII	$(\alpha1(XII))_3$	Chicken embryotendon, bovine periodontal ligament
XIII	Unknown	Cetal skin, bone, intestinal mucosa

As describe above, it is obvious that collagen is the structural protein which has various diversities including its structure, size, assembly mode and function as well as tissue distribution. Among all of the collagen types, type I collagen is predominant genetic product which is essential for many organs in keeping their tensile strength, form and cohesiveness. Collagen is usually found in tissue which has high tensile strength such as skin, tendon and blood vessels as well as bone (Albert, 2002). Like other collagens types, type I collagen consists of three polypeptide chains which is mostly α1 and α2. Nevertheless, heteromeric type (α1α2α3) and homomeric type $(\alpha 1)_3$ can be found as a minor form (Yamauchi, 2002). It has been found that most teleost skin such as cod and Alaska Pollack are consist of distinct subunit (α3) which can not be detected in mammalian collagen (Kimura et al., 1987a). In 1987b, Kimura and Ohno studied the type of α chain in type I Alaska collagen and found that the α3 was located only in specific tissue such as skin collagen but not in the swim bladder. Moreover, they suggested that the α3 polypeptide chain might be a product of a third genetic locus which arose as a duplication of α1 gene.

B. Physicochemical properties of collagen

Collagen is one of the reputable extracellular matrices applied in tissue engineering and medical products. Extracting method is based on its unique characteristics such as acid soluble protein, salt precipitation and enzyme resistance. The principal method widely used is extraction using diluted acid solvents (0.5 M acetic acid, citrate buffer, or HCl pH 2-3). The weak acids dissociate aldimine intermolecular crosslink of collagen and repulse the repelling charges on the triple helical molecules. These capabilities result in its fibrillar structure swelling. However, less labile crosslink like keto-imine bond that is not broken down by the weak acid explains why extraction of collagen from the tissue containing high level of this crosslink type such as bone, cartilage and old animal tissue give low yield (Friess, 1998).

Much higher yield can be achieved by taking an advantage from the fact that at below 20°C, the triple helices of collagen are relatively resistant to protease enzyme. In such condition, the enzyme is able to cleave the peptide bond only at the junction between helical and non-helical sites being near the keto-amine

crosslink, thus leads to the releasing of the soluble molecules. Pepsin is the enzyme mostly used for this purpose. Therefore, the resulting material is called "pepsin soluble collagen (PSC)". Antigenic region removal located on the non-helical section, provokes lesser immune response than acid soluble collagen. Although the enzymatic method has many advantages as describe above, its product has some undesirable qualities such as, fast biodegradation rate and low mechanical strength. The disadvantages are the result from low level of its molecular crosslink (Friess, 1998). Ogawa and co-worker (2004) reported that ASC of subtropical fish skin and scale had population of crosslink higher than PSC ones. However, the crosslink can be accomplished by using chemical (Usha and Ramasami, 2000; Angele et al., 2004; Song et al., 2006) For example adding of 1-ethyl-(3-3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) and N-hydroxysuccinimide (NHS) can generate the peptide like bond. The resulting product has an improvement of collagen biocompatibility, cellular differential potency and enzymatic degradation resistance (Angele et al., 2004). Moreover, the collagen can be stabilized by various substances such as glycerol (Penkova et al., 1999), amino acids (Changyou et al., 2004) and iron complex (Fathima et al., 2006).

It is obvious that collagen exhibits biodegradability, weak antigenicity and superior biocompatibility compared with other natural polymer like albumin or gelatin (Table 2.2). Even though it has many advantages, potential risk can occur. Collagen originating from bovine is capable of transmitting the disease like bovine spongiform encephalopathy (BSE). Therefore, the use of aquatic collagen can be served as an alternative source and fish is the most interesting collagen source. Many fish species have been put into consideration such as Nile perch (Muyonga et al., 2004), Japanese flounder (Nishimoto et al., 2005) and redeye snapper (Jongjareonrak et al., 2005). In addition, extracted sources are also diverse. In fishery processing industry, skin and scale are the most promising by-products with abundant amount of collagen. In 2000, Nagai and Suzuki extracted collagen from fish skin, bone and fin. They found that the highest yield can be obtained from fish skin and that fin is composed of collagen higher than bone. However, collagen in each fish species are varied [10-25.7% base on wet weight (Kittiphattanabawon et al., 2005 and Nishimoto et al., 2005) and 31-63.1% on the dry weight basis (Nagai and Suzuki 2000b; Mizuta et al., 2003; Muyonga et al., 2004)].

Although fish is plentiful of collagen, collagen obtained from this source has some drawbacks including low level of intra- and inter-molecular crosslink and low imino acid composition. Kittiphatanabawon and co-workers (2005) indicated that fish collagen is generally less stable than mammalian counterparts owing to its low imino acid contents resulting in its low thermal resistance. Furthermore, the upper limit of physiological temperature is also related to its denatured temperature. This finding helps explain why collagen extracted from subtropical and tropical fish have thermal stability higher than the cold water ones.

Table 2.2 Advantages and disadvantages of collagen as a biomedical product (modified from Lee et al, 2001)

Advantages	• Available in abundance and easily purified from living organisms (constitutes more than 30% of vertebrate tissues) • Non-antigenic • Biodegradable and bioreabsorbable • Non-toxic and biocompatible • Synergic with bioactive components • Biological plastic due to high tensile strength and minimal expressibility • Haemostatic –promotes blood coagulation • Formulated in a number of different forms • Biodegradability can be regulated by cross-linking • Easily modifiable to produce materials as desired by utilizing its functional groups • Compatible with synthetic polymers
Disadvantages	• High cost of pure type I collagen • Variability of isolated collagen (e.g. crosslink density, fiber size, trace impurities, etc) • Hydrophilicity which leads to swelling and more rapid release • Variability in enzymatic degradation rate as compared with hydrolytic degradation • Complex handling properties • Side effects, such as bovine spongiform encephalopathy (BSE).

C. Effect of collagen on dermal wound healing

Wound healing process is the complex mechanism involving many body systems including immune system. This process can be divided into three overlapping phrases, haemostasis and inflammation process, proliferation process and following maturation and remodeling process. When the wound occurs, platelets expose with subendothelial collagen resulting in an aggregation of the platelets and activation of the intrinsic part of coagulation cascade. Then, fibrin clots are formed. They are essential for temporarily sealing the wound, providing a lattice framework for various types of cell such as neutrophil, monocyte and fibroblast as well as endothelial cell. Furthermore, the following as an increasing in vascular permeability, releasing of prostaglandin and chemotactic substances including complement factors, interleukin-1, tumor necrotic factor-α (TNF-α), and transforming growth factor-ß (TGF-ß) as well as bacterial products, would stimulate neutrophil migration to the affected wound. Neutrophil migration is followed by macrophage, lymphocyte and fibroblast movement respectively. Wound macrophage derived from blood macrophage migrates into a wounded area. The macrophage plays an important role in inflammatory process, for example, their phagocytosis activity leads to the releasing of cytokines and nitric oxide. These substances have an impact on angiogenesis and fibroplasias. Fibroblast is also necessary for this mechanism. It is derived from undifferentiated mesenchymal cell and local tissue fibroblast. This cell migrates into the wound space in order to synthesize and deposit various glycoproteins and collagen. Its migration mainly results from macrophage derived cytokines such as TGF-ß, epidermal growth factor (EGF) and platelet derived growth factor (PDGF) (Swain and Henderson Jr., 1990).

After 2-3 days wounding, injured tissue would be in the proliferation process. Fibroblast and endothelial cell nearby the wound will begin to divide and multiply. These evidences are the results from various growth factors and cytokines derived platelet and activated macrophage. The endothelial cells form new capillaries and fuse with another vessel or endothelial bud and then blood flow begins. At this time, the white blood cells undergo apoptosis and they are digested by macrophage afterward. The connective tissue space initially filled with proteoglycans, protocollagen and tropocollagen begin to display clearly differentiation. The

component presenting in the wound space is called ''granulation tissue'' It is the mixture of branching capillary loops surrounded by mesenchymal cells and extracellular matrix. Tensile strength of wound develops during maturation and remodeling phases. Among wound healing processes, this process is the most important phase because level of scar strength depends on rate, quality and total amount of matrix deposition (Swain and Henderson Jr., 1990) (Table 2.4)

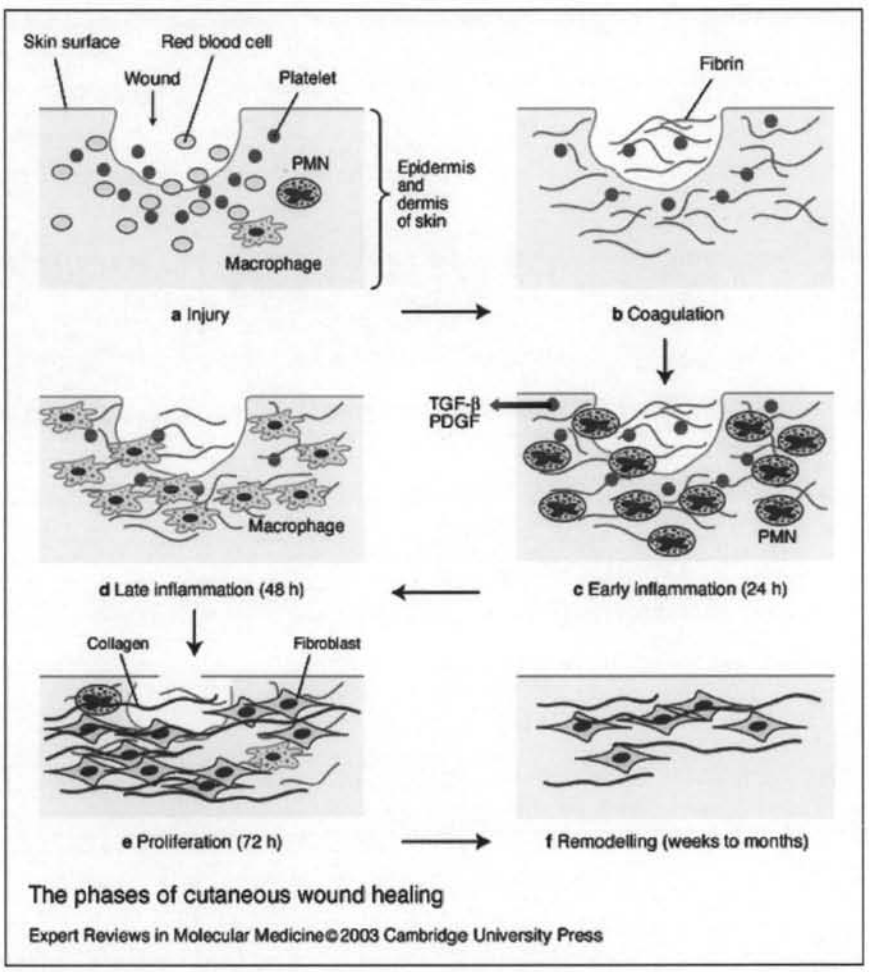

Figure 2.4 Wound healing mechanism (Steven et al., 2003)

As mentioned above, the wound healing process is associated with time and balanced activity of inflammatory, vascular and connective tissue as well as epithelial cell. All of which need an extracellular matrix to facilitate the healing process. In evolution of biologic and synthetic wound dressing, it is obvious that any

wound requires a barrier protection in order to prevent skin infection, desiccate the wound and be framework for cell attachment so as to maximize healing. Therefore, wound coverage with material containing epidermal and dermal analogues is widely accepted in medical use. The outer layer functions like epidermis acting as a barrier to protect the wound not only from bacterial infection, fluid loss but also overheating and accumulation of tissue fluids. In case of lower layer element, it is essential for cell guidance during granulation formation, remodeling and re-epithelialization phrases. The synthetic element acts like a scaffold for cell attachment (temporary substitutes) and then it will be incorporated in dermal element (permanent substitutes). The synthetic analogue is important for restoring normal tissue architecture and reducing the scar formation. The raw material of dermal layer is mostly derived from collagen or collagen combined with other extracellular matrices such as glycosaminoglycan, hyalulonic acid or fibroblast cell. Collagen is not only important for cell attachment, growth, and differentiation but also influential in desirable structural properties of various cells. Moreover, the extracellular matrix has many functional properties, for example, it can release the chemical substance which can attract and promote fibroblast proliferation and differentiation (Ruszczak, 2003).

The attachment of fibroblast and collagen matrix requires transmembrane cell adhesion protein called "integrin". It has function in binding the matrix to the cell's cytoskeleton. Integrins differ from other cell surface receptors for hormone and other extracellular soluble signal molecules in that they generally bind their ligand with low affinity. However, the number of them presenting at cell surface are higher than other cell receptors about ten to hundred fold. According to Velcro principle, cells can take advantage from their distinct characteristics, cells attach collagen with optimum force and motile simultaneously. Furthermore, integrins also play a role in communication between cell and collagen via activation of intracellaular pathway (Albert et al., 2002).

Integrin is a transmembrane matrix receptor composed of two subunits namely α and ß subunits. The structure of α subunit is a single transmembrane helix whereas ß transmembrane protein is a short intracellular tail (Figure 2.5). Because there are numerous subunit types, they can form various heterodimer types. Moreover, the binding of integrins to their ligands are also related to extracellular divalent cations of integrins such as Ca^{2+} or Mg^{2+}. These cations are the one factor that

has an impact on the affinity and specificity of their binding (Tuckwell and Humphries, 1996).

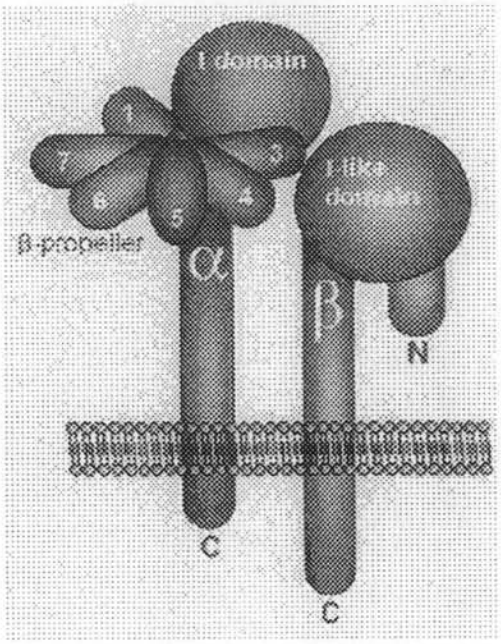

Figure 2.5. Schematics of integrin structure (Gullberg and Lundgren-Akerlund, 2002)

On the other hand, the extracellular marix has specific region for integrin to recognize called Arg-Gly-Asp (RGD). The RGD is the minimal recognition sequence of extracellular molecules. The RGD is found in α1 and α2 chain of type I collagen. The major type of fibroblast surface receptor for types I collagen are α1ß1, α2ß1 and α11ß1 (Table 3). As integrin interacts with collagen, cytoplasmic protein tyrosine kinase namely FAK (focal adhesion kinase) is activated. The focal adhesion protein activation promotes cell proliferation through mitogen activating protein kinase activation (MAPK) (Gullberg and Lundgren-Akerlund, 2002). Moreover, RGD can increase the level of intracellular calcium via tyrosine kinase activation. The increased in calcium ions is due to activation of the phospholipase C- phosphatidylinositol - Diacylglycerol (DAG)-IP3 (inositol 3,4,5 triphosphate) system. These activation pathways of integrin result in diverse effects of cell adhesion, migration and gene expression as well as cell proliferation (Mineur et al., 2005).

Table 2.3 Domain integrin ligands (Gullberg and Lundgren-Akerlund, 2002)

Integrin	Distribution in vivo	Extracellular matrix ligands
$\alpha 1\beta 1$	endothelium, smooth muscle fibroblasts	collagens (collagen IV > collagen I, collagen XIII), collagen C-propeptide, laminins, matrilin-1
$\alpha 2\beta 1$	epithelia, fibroblasts	collagen (collagen I > collagen IV), collagen C-propeptide, chondroadherin
$\alpha 10\beta 1$	cartilage	collagens
$\alpha 11\beta 1$	non muscle mesenchyme fibroblasts	collagen (collagen I > collagen IV)