CHAPTER III

LITERATURE REVIEWS

In this chapter, literature reviews are summarized into 2 parts as follows:

3.1 Influence of xenogeneic scaffold on tissue engineering application.

3.2 Preparation and characterization of acellular dermis.

3.1 Influence of xenogeneic scaffold on tissue engineer application.

In 2004, Stephen and coworkers [5] reviewed the benefits of xenogeniec scaffold for tissue reconstruction. ECM, typically from a xenogeneic source, has been used as a scaffold for the replacement or reconstruction of many different tissues. ECM is derived from organs such as the small intestine, urinary bladder or skin. These scaffold remains natural components such as collagen, fibronectin, glycosaminoglycan (GAG) and elastin. ECM components appear to be among the first and most critical ECM factors in the process of cell and tissue differentiation. An allogeneic or xenogeneic source of ECM has usually been used, thus providing the possibility of an off-the-shelf product for clinical application. There are differing opinions as to whether the ECM should be seeded with site-specific cells prior to utilization as a repair scaffold or should be used in an acellular form. The effect of adding cells to an ECM scaffold upon the biologic remodeling response varies depending upon the intended application and the manner of preparation of the composite scaffold. A cellular component requires an autologous cell source and a controlled environment to assure cell viability during the translocation of the cell-scaffold composite to the recipient tissue site. Both cellular and acellular forms of ECM scaffolds have been used for tissue engineering applications.

Scaffolds derived from xenogeneic extracellular matrix have found utility in tissue engineering application. The safety and efficacy of such scaffolds when used for the repair and reconstruction of numerous body tissues including musculoskeletal, cardiovascular, urogenital and integumentary structures has been shown in both preclinical animal studies and in human clinical studies. More than 200,000 human patients have been implanted with xenogeneic ECM scaffolds. These ECM scaffolds are typically prepared from porcine organs such as small intestine or urinary bladder, which are subjected to decellularization and terminal sterilization without significant loss of the biologic effects of ECM. The composition of these bioscaffolds includes the structural and functional proteins that are parts of native mammalian extracellular matrix. The three-dimensional organization of these molecules distinguishes ECM scaffolds from synthetic scaffold materials and is associated with constructive tissue remodeling instead of scar tissue. Xenogeneic scaffold are usually porcine-derived and elicit a constructive remodeling response that is dependent upon many factors. These factors include angiogenesis, support of host parenchymal cell attachment, proliferation and differentiation, and biologic responses that are mediated by degradation products of ECM scaffold itself. Future applications of ECM scaffolds in tissue engineering will likely depend upon the ability to add specific cell types (presumably autogenous) to the scaffolds prior to, or simultaneous to, implantation and the development of strategies for vascularization of three-dimensional ECM.

3.2 Preparation and characterization of acellular dermis.

In 1995, Takami and coworkers [4] developed a new method for preparing acellular allogeneic dermal matrix and studied its effectiveness as a dermal substitute. Donor skin was treated with either 0.25% trypsin or with 2.5 U/ml dispase II at 4°C for 24-48 hr to remove epidermis and cellular components from dermal matrix. Subsequently, the dermal matrix was incubated in 0.5% Triton X-100 at room temperature for 24 hr. The results showed that, treatment with trypsin for 48 hr removed most intact cells in dermis but cellular debris still remained in hair follicles and the structure of collagen was disrupted. Dispase II more effectively removed cellular components from skin than trypsin but neither enzymatic treatment alone was

sufficient to remove all cells and cell debris. The optimal conditions for preparing a cell-free dermal matrix with minimal damage to collagen structure required a treatment with 2.5 U/ml dispase II for 24 hr at 4°C followed by 0.5% Triton X-100 for 24 hr at room temperature. Subcutaneously implanted ADM evoked no immunological reaction and 20 weeks after implantation, the size of implanted ADM was reduced to about 60% of its original area. ADM became completely vascularized within 2 weeks after implantation into full thickness skin defects in a rat and inhibited extensive wound contracture. A second layer of ADM placed onto the implanted ADM served as an excellent dressing, providing mechanical protection and permitting vascularization of underlying implant. It was noticed that, dispase/detergent treated ADM derived from animal or human skin may be useful in full thickness skin defects providing a vascularized bed for subsequent dermal coverage.

In 1996, Medalie and coworkers [13] developed the composite grafts of cultured human keratinocytes and acellular human dermis. ADM was prepared by rapidly freeze-thaw in liquid nitrogen for 3 cycles and then soaked in PBS for 5 weeks to remove cellular component. After this, culture keratinocytes were seeded onto the surface of ADM, and the composite grafts were maintained submerged for 1 week prior to grafting. Grafts were transplanted onto athymic mice and studied up to 8 weeks. The results showed that, after 1 week of implantation human keratinocytes had formed a stratified epidermis. Composite grafts with keratinocytes were white and shiny with no apparent pigmentation. Histological of composite grafts revealed that, mouse fibroblasts were infiltrated into acellular dermis as early as 1 week. After 8 weeks, fibroblasts had completely repopulated the dermis, and blood vessels were evident in the most superficial papillary projections. Dermal element, such as elastin fiber, which was presented in the starting dermis, persisted for the duration of the experiment. Their results demonstrated that acellular dermis provided a suitable support for the seeding of cultured keratinocytes in vitro and a durable material that could be surgically secured in an open wound. In addition, composite skin grafts of culture keratinocytes on acellular human dermis can be successfully transplanted to full thickness wound on athymic mice.

In 1998, Walter and coworkers [11] studied the final composition of ADM prepared by two different methods. They have treated cadaver skin with either dispase II for 24 hr followed by Triton X-100 for 24 hr or NaCl for 24 hr followed by SDS for 24 hr. They showed that cellular components could be completely removed. The ADM samples were cryosectioned and then characterized immunohistochemically. Staining for cell-associated antigens (HLA-ABC, HLA-DR, vitamin, desmin, talin), ADM components (chondroitin sulfate, fibronectin, laminin, hyaluronic acid, elastin, and type VII collagen) was dramatically reduced or absent from ADM prepared by both methods. However, significant amounts of elastin, keratin sulfate, laminin, and type III and IV collagen were still observed in both ADMs. Both methods of ADM preparation resulted in extensive extraction of both cellular and extracellular components of skin but retention of basic dermal architecture. In general, ADM prepared by NaCl-SDS treatment retained larger amount of ADM components than that prepared by dispase-Triton treatment. The former may more readily support cell attachment and proliferation but may also exhibit more antigenicity upon implantation into wounds than Dispase-Triton treated ADM.

In 2003, Qian and coworkers [14] evaluated the growth rate and cell activity of cultured keratinocytes on acellular porcine dermis in order to develop a composite skin in vitro for burn injuries or other skin defects. Full thickness skin was cultivated from neonatal SD rats, and separated into epidermal layer and dermal layer with enzyme digestion. Acellular porcine dermis was prepared by enzymatic treatment. The dermis was treated with 0.25% trypsin/EDTA for 24 hr to remove cells. The keratinocytes were then seeded on the prepared acellular porcine dermis soaked in a culture medium. The cultures were incubated and the growth status of keratinocytes on acellular porcine dermis was evaluated by phase contrast microscopy, histological examination with hematoxylin–eosin staining and acridine orange staining, immunohistochemistry, observation of growth curve plotted by MTT colorimetry and analysis of changes in keratinocytes proliferation cycle with flow cytometer. The results showed that, almost all keratinocytes anchored in 48–72 hr, and most inosculated at days 6 and 7. The growth curve showed that the keratinocytes grew in logarithmic phase at days 3–6 after seeding. More than four layers of keratinocyte

structure and the basement membrane between keratinocytes and porcine dermis were observed. Pancytokeratin was strongly positive in the cultured keratinocytes. Laminin and type IV collagen were positive in the basement membrane. The conclusion was that the cultured keratinocytes on acellular pig dermis grow well and the structure of composite skin which has been established is satisfactory.

In 2004, Chen and coworkers [12] compared decellularization at each critical step in the processing of ADM from porcine skin. In order to study the effect of each critical step, the porcine skin was divided into four groups. Group A was processed to compare the influence of treatment conditions of the trypsin solution on the extent of decellularization. Group B was designed to compare the influence of washing time with 0.1% SDS solution on the extent of decellularization. Group C was processed to compare the influence of treatment conditions of both trypsin and dispase on the extent of decellularization after removing the epidermis of porcine skin. Porcine skin in group D was designed to compare the influence of washing time of the dispase solution and SDS solution on the extent of decellularization. The results demonstrated that the removal of epidermis using a treatment with 0.25% trypsin for 18 hr and 0.1% sodium dodecyl sulfate (SDS) for 12 hr at room temperature was beneficial for the subsequent treatment to remove cells in the dermal structure. Lengthy incubation in 0.25% trypsin (12 hr) and then 560 units/l dispase II (12 hr) at $25^{\circ}C$ of small pieces of porcine skin, from which the epidermis had been removed, efficiently removed cells and cellular components. To achieve the complete removal of cells originally presented in the dermal matrix, the process cannot be simplified by shortening the treatment time. The optimal conditions in this study were defined that de-epidermis using a treatment with 0.25% trypsin at $25^{\circ}C$ for 18 hr, then followed by incubation with 0.25% trypsin at $25^{\circ}C$ for 12 hr, washing with 0.1% SDS at room temperature for 12 hr, treatment with 560 units/l dispase at room temperature for 12 hr, and a final washing with 0.1% SDS at room temperature for 12 hr. Histological examinations revealed that the epidermis, dermal fibroblasts, and epidermal appendages were completely removed by these treatments, and the basic dermal architecture of collagen bundles was that of a loose meshwork. Examinations by TEM and SEM showed that the characteristics of collagen fibers in the ADM were retained after complete

removal of cells under optimal conditions defined in this study. SDS-PAGE revealed that collagen fibers in the ADM were mostly type I. Size-exclusion HPLC showed two typical component peaks identified as oligomers and monomers.

In 2005, Wang and coworkers [15] produced an artificial skin composed of a crosslink silicone sheet on the surface of acellular porcine dermis which they have called silicone acellular porcine dermis (SAPD). This new artificial skin can protect the wound long enough to promote wound healing either by second intention or covered long enough until cultured epithelium autograft (CEA) or autologous skin graft can be harvested for permanent coverage. They delivered 4 cm x 5 cm full-thickness wound on the back of Sprague–Dawley rats. Thirty-six rats were divided into two groups. Eighteen rats had SAPD and the other 18 were covered with Biobrane. The wounds were first examined 2 weeks after grafting and followed weekly for an additional 4 weeks to evaluate the wound and study pathological changes by using H&E and Masson's stains. Wound size was calculated by a ruler and analyzed by statistic t-test. At the 2-week inspection, both SAPD and Biobrane showed tight adherence to the wound with no change of wound size. Both SAPD and Biobrane dermal templates were pink. In the Biobrane-covered group, the wounds contracted soon after the tie-over dressing was removed. Its dermal layer is a layer of thin porcine dermal substance, which was promptly digested by tissue hyaluronidase and provides no real dermal template. In the SAPD-covered group, however, the wound size was maintained significantly from third to sixth week after grafting. SAPD was designed with thick epidermal silicone and a well-organized porcine dermis so that it incorporates into the recipient wound. Clinically the silicone layer of SAPD dislodged from SAPD about 6–7 weeks after grafting and was followed by dermal matrix exposure and infection. In pathological examination, much like a human skin graft, new vessels were found in SAPD about 1 week after grafting with minimal inflammatory cells infiltrated in the graft and wound. Six weeks after grafting, the SAPD incorporated into the wound, showing palisade arrangement and no sign of rejection. In the Biobrane group, the wounds showed severe inflammation, the porcine dermal matrix was digested and disappeared 3 weeks after coverage. In conclusion, SAPD is a thick biosynthetic artificial skin, which protects the rat wound

significantly longer than Biobrane and prevents contraction.They expected that using of SAPD for temporary wound coverage will provide enough time to grow autologous cultured epithelium or to reharvest skin grafts.

In 2006, Gilbert and coworkers [8] reviewed the decellularization protocols of tissues and organs. The efficiency of cell removal from a tissue is dependent on the origin of tissues and specific methods used. 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 treatment or ionic solutions, followed by separation of cellular components from ECM using enzymatic treatments, solubilization of cytoplasmic and nuclear cellular components using detergents, and finally removal of cellular debris from the tissues. 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 the chemicals. The efficiency of decellularization and preservation of ECM can be assessed by several methods. The mechanisms of physical, enzymatic, and chemical decellularization for a variety of tissues are reviewed in Table 3.1

Table 3.1 Commonly used decellularization methods.

Methods	Mode of action	Effects on ECM
Physical		
Snap freezing	Intracellular ice crystals disrupt cell membrane	ECM can be disrupted or fractured during rapid freezing
Mechanical agitation	Can cause cell lysis, but more commonly used to facilitate chemical exposure and cellular material removal	Aggressive agitation or sonication can disrupt ECM as cellular material is removed
Chemical		

Alkaline; acid	Solubilizes cytoplasmic components of cells; disrupts nucleic acids	Removes GAGs
Triton X-100	Disrupts lipid–lipid and lipid–protein interactions, while leaving protein–protein interactions intact	Mixed results; efficiency dependent on tissue, removes GAGs
Sodium dodecyl sulfate	Solubilize cytoplasmic and nuclear cellular membranes; tend to denature proteins	Removes nuclear remnants and cytoplasmic proteins; tends to disrupt native tissue structure, remove GAGs and damage collagen
EDTA, EDGA	Chelating agents that bind divalent metallic ions, thereby disrupting cell adhesion to ECM	No isolated exposure, typically used with enzymatic methods
Enzymatic		
Trypsin	Cleaves peptide bonds on the C-side of Arg and Lys	Prolonged exposure can disrupt collagen structure, removes laminin, fibronectin, elastin, and GAGs