Screening Models for Tissue Engineering

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5.1 General Overview

5.1.1 Roles of Screening Models in the Development of Devices for Oral and Maxillofacial Regeneration

In vivo tests of the tissue response to implants represent just one facet of the evaluation of candidate implant materials/devices. The myriad tests included under the heading of "biocompatibility" reflect the wide variation in the different components to the host response to implants. Implantation tests can be employed to assess the efficacy of devices (functional effectiveness of devices) as well as the safety of the materials of fabrication. The term "biocompatible" is employed to describe a wide variety of (generally *in vivo*) findings, from a benign tissue response to a biomaterial in an animal model to the successful clinical implementation of a medical device. Moreover, the term is also often used to generally describe biomaterials which have met certain standards of safety and efficacy in select situations. While there is convenience in using the term "biocompatibility" to convey a general assessment of a biomaterial or medical device, its nonspecific use can be misleading. Its use should be qualified with respect to the specific conditions under which the biomaterial/device is being evaluated. The device should be considered biocompatible only in the context of the criteria used to assess the acceptability of the tissue response in relation to the required function of the device.

This chapter will deal with select protocols that have been found to be of value for the initial *in vivo* screening of implants. While most of these implants comprise nonresorbable biomaterials, they can be adapted for use with scaffolds for tissue engineering and regenerative medicine and for tissue-engineered constructs. Preclinical tests required for qualification of the device for human trial are generally directed toward the evaluation of the device in animal models – often using large animals – that approach the specific clinical application in

which the device is to be used. Because these preclinical test protocols, which are the subjects of other chapters in this book, are expensive and time-consuming, it is important to prescreen the device in less complex animal experiments, which can provide an indication whether the next step of preclinical testing is warranted.

Experiments involving the implantation of materials into animal tissues date back to the early 1800s. The level of interest in devising more effective chronic implantation protocols has increased significantly during the last 20 years. This is due to:

- The need for new materials with properties that satisfy the ever-increasing functional demands of implant devices
- The availability of new candidate implant materials, especially the large number of tissue engineering products
- The increasing awareness of the complexity of the biological response to biomaterials.

Most recently there has been an increased awareness that standardized screening protocols need to be developed. Such standardized protocols are considered by many as essential for "evidence-based medicine" and to facilitate the requirements of federal regulatory agencies. The elements of the screening protocol include the:

- Species: gender and age
- Site of implantation
- Shape of the implant
- Period of implantation
- Method of evaluation of the tissue response
- Criteria for determining if the response in the animal model is potentially harmful to the human host.

The problematic aspects of this type of testing include:

- Identification of an experimental animal and site of implantation that will serve as an adequate model of the human response
- The selection of a shape of implant and site of implantation that will adequately distinguish the response elicited by the chemical formulation of the specimen from tissue responses to the implant shape, mechanical trauma associated with the very presence of the implant, and the wound healing processes
- The selection of criteria to determine if the animal response can be used to predict whether the material can be safely used in the human host.

Despite these challenging aspects of implantation testing, there has been no disputing the implementation of screening protocols to assist in the initial determination of the safety and potential efficacy of candidate implant materials.

Tissue engineering concepts for oral and maxillofacial regeneration require appropriate models to test osteogenic devices. A strategic approach to preclinical testing is needed to determine the *in vivo* performance of material devices for oral wound healing. There is a significant complexity to the healing *in vivo* compared with the *in vitro* environment. There is the need for various studies on a particular biomaterial, biologic agent or device including safety and efficacy studies in multiple models prior to proceeding to Phase I human clinical trials.

There are multiple factors that affect osseous bone healing that are best evaluated in the *in vivo* environment, including biomechanical, cellular, and vascular mechanics that comprise the healing process. The models chosen to assess a particular device should mimic the environment in which the device will be used therapeutically. One should choose a "critical-size defect" that will not spontaneously regenerate, to best characterize the contribution of the device to healing. Tissue engineering devices can be screened in various preclinical animal models to determine their potential. Depending on the knowledge base and previous data on a product one can choose the appropriate model to

screen the potential of a new device. For new technology it is advised to begin with small animal models which can provide early data in a relatively fast and cost-effective way. The research can then progress to large animal wound systems that simulate more closely the human wound and therapeutic environment, more closely associated with the planned clinical application of the device.

The goal of this chapter is to provide a stepwise "roadmap" for evaluating potential biomaterials to be utilized for bone healing such as: osteoconductive or osteoinductive systems, either passive or bioactive scaffolds, and biomimetic systems. It is necessary to demonstrate adequate safety and efficacy prior to moving to more complex preclinical models, and it is advised to gather adequate data from small animal models prior to investing economically in large animal model research.

5.1.2 Historical Perspective on Implantation Protocols

While a chronological review of the implant literature can be instructive in revealing how an understanding of the tissue response to materials evolved, it can also be confounding. Tissue responses observed in experimental studies have varied considerably with the animal model, site of implantation, and shape of the implant. For example, while no adverse response was elicited by a cobalt-chrome specimen implanted in bone, fibrosarcoma was found adjacent to films of the same material implanted in subcutaneous sites in the rat. Results of this type evidence the need for some degree of standardization of screening protocols. Only a few studies reported in the literature have had as their objective the development of an implantation protocol to be used in the assessment of the safety of candidate implant materials. Most implantation investigations have had as their focus the tissue response elicited by specific materials. The choice of the site of implantation was most often determined by the projected use of the material. Little rationale beyond the availability and ease of handling was generally given for the choice of animal model. Routine histopathological examination of tissue adjacent to the implant material was most often the method of evaluation of the biologic response. Generally very little discussion was directed toward the influence of implantation time or size and shape of the implant on the biologic response. In addition, little mention was made of the criteria that might be appropriate for interpreting the changes observed in the host tissue adjacent to the implant. Nevertheless these studies have generated the data which serve as the foundation for an understanding of subacute tissue responses to biomaterials. The protocols that we now accept as standardized screening tests are those specific protocols which have been "validated" through repeated use by different groups. Their utility has been demonstrated in the consistent results that they generate.

5.1.3 Determinants Underlying the Tissue Response to Implants

Determinants of the tissue response to biomaterials, and the subsequent assessment of biocompatibility, reside in the intrinsic wound healing process in the particular tissue or organ into which the biomaterial has been implanted. This wound healing process, initiated by the surgical trauma of implantation, can be modulated by: the physical presence of the device, and its porosity and modulus of elasticity; and the chemical composition of the material and agents released by the biomaterial. The tissue response to a biomaterial is, therefore, a characteristic of the host tissue's wound healing process as well as being a characteristic of the biomaterial. There is often an implication that a biomaterial which displays a favorable (biocompatible) response in a particular test (i.e., tissue) has met the condition of biocompatibility for all applications in which it may be used. In light of the above, the tissue response to the

same biomaterial might be quite different depending on the tissue in which it is implanted. Biocompatibility is not, therefore, an invariant property of a biomaterial.

5.2 Models for Bone and Soft Tissue Implants

5.2.1 Extraoral Bone Defects

Screening protocols have been employed for decades to assess the osseous response to the chemical make-up, pore structure, and surface features (at the nanometer, micrometer, and millimeter scales) of implants. These studies have been directed to evaluate the degree to which implants fabricated from certain biomaterials and with surface features become osseointegrated, and the amount of bone that forms within surface and internal pores (i.e., "bone ingrowth") of porous implants. The results, quantitatively, can be influenced by the type of bone into which the implant is placed, e.g., cortical versus cancellous, based on the available pools of osteoprogenitor cells available to participate in the bone regeneration process.

5.2.2 Advantages of the Circumscribed Defect Model in Long Bones

One of the first protocols to be used systematically (Melcher and Irving, 1962) to compare the osseous response to a variety of biomaterials involved implantation into cylindrical (circumscribed) defects through the cortex and into the medullary canal of the femoral diaphysis of rats (Melcher and Irving, 1962), rabbits, and dogs (Spector *et al*., 1976).

One of the advantages of this model is that the response of both cortical and cancellous bone to the implant can be evaluated. In addition, for larger animals, such as dogs, goats, and sheep, six implants can be implanted into each femur. Moreover, an option to harvesting all of the implants at the time of sacrifice is to biopsy select implants at various time periods post-implantation (Spector *et al*., 1976). These aspects of the consideration of the critical-size defect model have been under scrutiny over the past years (Horner *et al.*, 2010; Reichert *et al.*, 2009).

5.2.3 Surgical Procedure in the Circumscribed Defect Model in Long Bones

A longitudinal incision is made in the skin overlying the lateral aspect of the femur. The thigh muscles are separated and the lateral aspect of the femur is exposed. The holes are produced with a slow-speed power (e.g., dental drill) or hand drill, operated under ample irrigation for cooling. The diameter of the holes and depth are adjusted to the diameter of the femur. For example, for rats the hole diameter is 2 mm, and for dogs the holes are 4 to 6 mm in diameter. The medullary cavity is exposed, the wound irrigated with saline, and the implants inserted with a press-fit. In order to assure a proper interference fit of the cylindrical implant in the hole, a fluted reamer precision-machined to match the diameter of the implant can be used after the hole is prepared with the twist drill. After insertion of the implant the soft tissues are closed over the hole.

If the plan is to biopsy select implants prior to sacrifice, a trephine with a diameter 1 to 2 mm larger than the diameter of the implant can be used to remove the implant with a collar of surrounding bone. One difficulty with this procedure is that the bone regeneration process is generally so rapid and complete that it is difficult to precisely identify the location of the implants in the diaphysis. One option is to use a custom-designed drill guide to ensure reproducibility of the location and size of the defect and subsequent boring of the material within the defect after the designated implantation time (Orr *et al*., 2001). The drill jig is held onto the bone with two stainless steel guidewires and the guide hole used to locate the drill bit at the desired site of

implantation. After the hole is drilled the jig is removed but the two stainless steel pins are left in the bone. At the time of the harvest, the corresponding recovery jig is fixed on the two stainless steel pins. The hole in the recovery jig is sized to accept the trephine used to core bore the implant and surrounding bone from the site of implantation.

5.2.4 Methods of Evaluation of the Circumscribed Defect Model in Long Bones

The circumscribed bone implant protocol lends itself to several methods of evaluation, including: mechanical testing, micro-computed tomography (CT), microradiography, and histology. The resected samples can be trimmed to enable mechanical test of the push-out strength (Chang *et al.*, 2010a; Chang *et al.*, 2010b). The bone surrounding the implant can be supported in a jig in a mechanical test machine and a plunger placed on the implant to enable a compressive load to be applied to push the sample out of the bone. The load to failure can be divided by the estimate of the implant–bone interfacial surface area to yield the interfacial shear strength. Specimens of the implant and surrounding bone which remain undecalcified can be (formalin) fixed immediately after resections from the bone or after mechanical testing and dehydrated and embedded in a plastic embedding resin. Sections approximately 100 µm can then be sawn from the blocks. These sections can be placed on high resolution X-ray film or digitized plate to yield contact radiographs with less than 1 mm resolution of bone features (i.e., microradiography). The 100 µm sections can subsequently be ground to about 40 µm and surface stained (e.g., with toluidine blue) and examined under a light microscope for "ground section histology". Alternatively the samples resected from the bone can be fixed, decalcified, dehydrated, and embedded in paraffin for histological evaluation. These same methods of evaluation can be employed for implants in other sites, which are described below.

5.2.5 Mandibular Symphyseal Defect Model and Ramus Trephine Defect Model

Extraoral incisions are utilized to access the mandible for the use of the mandibular symphysis or the ramus for implantation. The ramus trephine defect creates a through-and-through defect due to the limited thickness of the ramus bone. The use of a trephine drill creates a critical-size defect that will not heal spontaneously, but which fills spontaneously with fibrous tissue. This defect lends itself to early testing for osteogenic response to a biomaterial implant.

For implantation into the ramus, an oblique incision is made over the ramus and the tissues are elevated to access the bone surface with full thickness blunt dissection. The defect is then created utilizing a trephine with rotary instrumentation. The implant is placed *in situ* and then the site is closed in two layers: first the muscle layer with internal absorbable sutures, and then the surface incision is sutured.

5.2.6 Calvarium Standardized Defect Model

Numerous studies have employed defects in the calvarium as the site in which to screen biomaterials for the bone response that they elicit, principally because the diameter of the critical-size defect is smaller than in long bone sites. Another advantage is that multiple defects can be produced in the same surgical field. An incision is made through the scalp to expose the periosteum, which is then elevated and retracted to expose the bone. Defects can be produced with standardized trephine burs, using continuous saline irrigation for cooling. The biomaterials can then be placed into the defects (Cooper *et al.*, 2010).

5.3 Models for Bone and Soft Tissue Implants

5.3.1 Soft Tissue Sites for Implantation – The Ectopic Bone Model

Several soft tissue sites of implantation have been proposed for screening protocols. The paravertebral muscle has been proposed because of its advantage of being large enough to host at least three implants per muscle (Coleman *et al*., 1974). In addition, this muscle provides a homogeneous implant environment and is easily accessible surgically. The disadvantage of the paravertebral muscle, however, is that it is mechanically active.

Sites in the subcutaneous tissue of rats and rabbits, and in the paravertebral muscles of the back of rabbits (Coleman *et al*., 1974), are sites frequently used to evaluate the soft tissue response to implants. For these procedures, the animals are positioned such that the operative site on the back of the animal can be shaved, and the skin cleaned and prepared for the sterile procedure. Sterile drapes are placed around the surgical area and attached to the skin with staples; 1 cm long incisions are made on each side of the spine, approximately 1.5 to 2 cm from the midline. Two subcutaneous pockets, one rostral and one caudal with 2 cm spacing, are made in each incision by blunt dissection. Four implant sites are thus prepared on the back of each animal and have also been demonstrated for other small animals such as mice and rats (Jin *et al*., 2008).

For intramuscular sites in rabbits, four longitudinal incisions about 1 cm long are made on the back of the animal. One superior and one inferior incision, located by the thoracic 9 to 11 and lumbar 3 to 4 spinous processes, are placed 1.2 to 2 cm apart on each side of the spine. The subcutaneous tissue and fascia are incised, and the tissues retracted to expose the underlying muscle. The muscle incision is sutured using 3-0 resorbable suture, and the skin incisions are closed using 4-0 interrupted suture.

5.3.2 Ectopic Bone Models for Screening of Osteogenic Molecules

Such ectopic bone models are useful for the screening of osteogenic molecules for proof-of-concept studies for bone regeneration (Anusaksathien *et al*., 2004). Figure 5-1 to Figure 5-5 provide examples of using such constructs with a variety of different carrier devices (e.g., polymeric or ceramic) for characterization macroscopically, histologically or using micro-CT. Further, in addition to the evaluation of pure bone wound healing models in the ectopic sites, more recently the evaluation of bone-tooth-ligament constructs can also be assessed with a variety of imaging methods (Park *et al*., 2010). The specifics for these animal models are described elsewhere (Anusaksathien *et al*., 2004; Park *et al*., 2010).

5.4 Recommended Practices for *in vivo* Screening from Standards-Writing Organizations

The development of standardized screening protocols is of obvious importance and has been the subject of previous reports in the literature extending over the past few decades. One such review (Coleman *et al*., 1974) proposed that certain basic criteria should be met by any implantation study.

- The elimination and/or standardization of variables which might affect the tissue reaction to the material.
- Adequate controls to eliminate the variability of the reaction due to unusual or unique individuals in the test population.
- Test animals should be relatively inexpensive and easily cared for.
- Implantation sites should be easily accessible surgically, provide a homogeneous environment for the implant, and be mechanically inactive as possible (unless mechanical trauma is being studied).

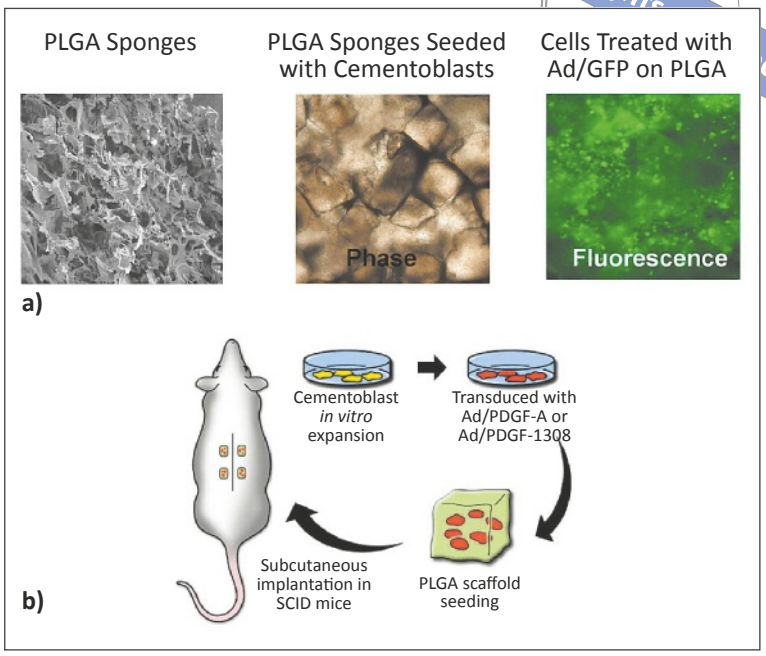

Fig 5-1 Engineering of hard tissues (bone or cementum) in three-dimensional polymer scaffolds. (**a**) Images depict microscopic structure of poly(lactic-*co*-glycolic acid) (PLGA) scaffold and cells seeded into the scaffold. The polymer scaffold exhibits open-pore structures allowing cell penetration and attachment. A representative phase contrast image depicts the polymer seeded with cells transduced with a control vector 48 hours after transduction. The fluorescent image shows the corresponding cells expressing green fluorescent protein (GFP) in the scaffold. (**b**) Diagram depicts transduced with adenovirus encoding growth factor transgenes, or no treatment, 24 hours after transduction. The transduced cells were seeded into polymer scaffolds and implanted into immunodeficient (severe combined immunodeficiency [SCID]) mice. PDGF, platelet-derived growth factor. Reprinted from Anusaksathien *et al*. (2004), with permission from the American Academy of Periodontology.

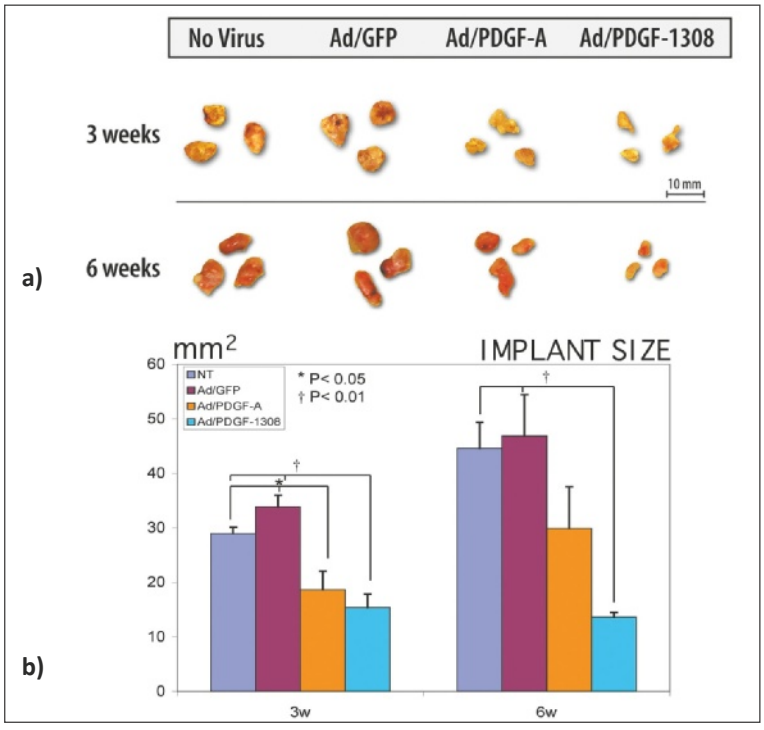

Fig 5-2 Macroscopic appearance and size of retrieved tissue engineered implants. (**a**) Standardized image shows the macroscopic appearance of polymer-cell implants following gene transfer of Ad/GFP, Ad/PDGF-A, AD/PDGF-1308 or NT, at 3 and 6 weeks post implantation. The bar represents 10 mm. (**b**) Histomorphometric analysis of the peri-implant areas (mm^2). GFP, green fluorescent protein; PDGF, platelet-derived growth factor; NT, no treatment. Reprinted from Anusaksathien *et al*. (2004), with permission from the American Academy of Periodontology.

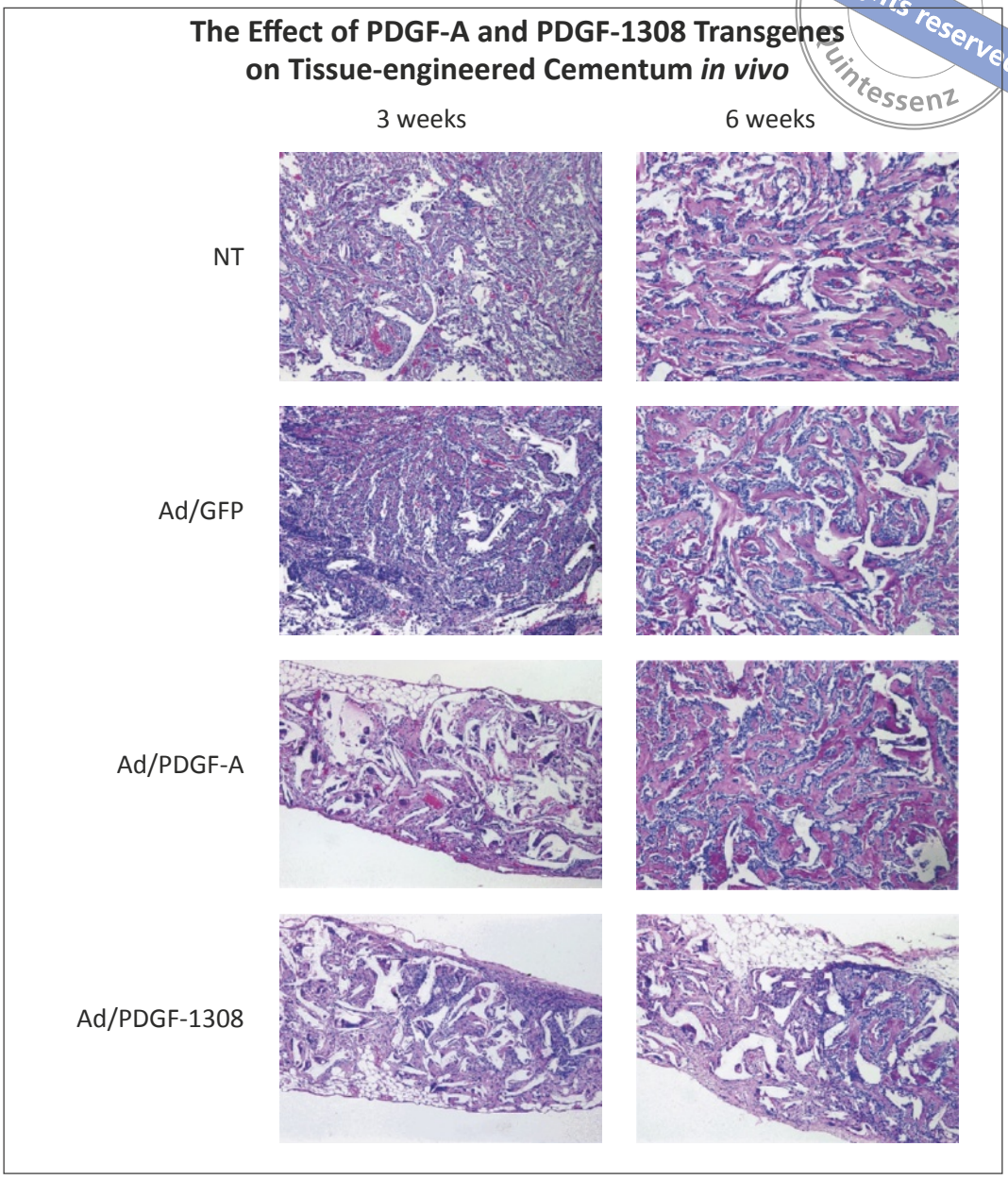

Fig 5-3 The effect of growth factor transgenes on tissue-engineered mineralized structures at 3 and 6 weeks *in vivo*. Mineralization was minimal to none in the Ad/PDGF-A and Ad/PDGF-1308 treated implants, whereas immature mineral formation was present in the NT and Ad/GFP implants at 3 weeks (left panels). Mineral formed was laced-like, with no hematopoietic or fatty tissue. At 6 weeks, mineral formation progressed in all groups, except the Ad/PDGF-1308 specimens, where minimal mineral formation was noted (right panels). (Hematoxylin and eosin, ×100 magnification). GFP, green flourescent protein; PDGF, platelet-dervied growth factor; NT, no treatment. Reprinted from Anusaksathien *et al*. (2004), with permission from the American Academy of Periodontology.

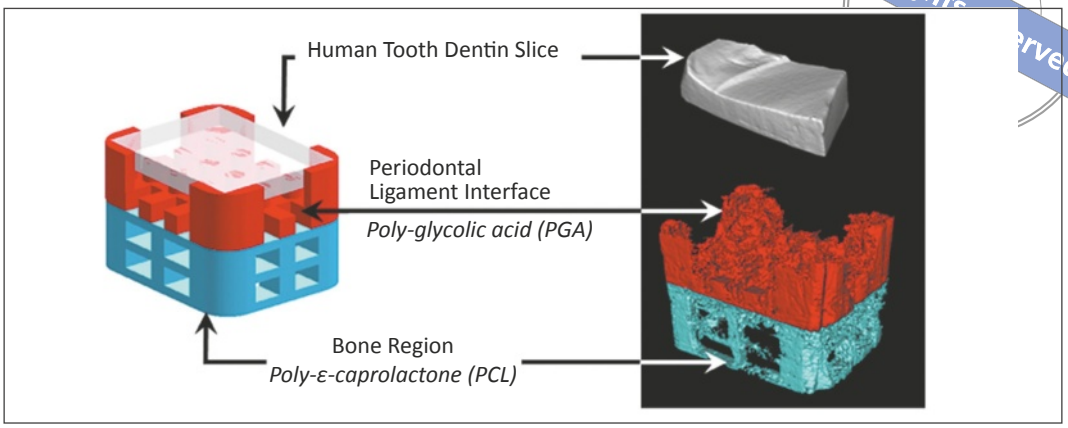

Fig 5-4 Schematic illustration of the modeling and dimensions of hybrid scaffold shows the procedure of polymeric architecture manufacturing for *ex vivo* engineering of tooth-ligament-bone constructs. After acid-treatment of human tooth dentin slices, the complex with a polymer-casted hybrid scaffold and a human tooth dentin slice was made using fibrin gel with or without cells. The right panel is the three-dimensional micro-CT scanned and reconstructed hybrid scaffold and a dentin slice. Reprinted from Park *et al*. (2010), with permission from Elsevier.

- Proper sterile and pyrogen-free techniques should be used during implantation.
- Experimental design should be such that results are reproducible within experimental limits.
- Ideally the results should be quantitative rather than purely qualitative.

Over the years, several organizations have sought to develop standardized test protocols which include as one of their components recommended practices for assessing the long-term (chronic) tissue response. At the time that the protocols were developed the intent was to screen permanent biomaterials for their "biocompatibility", but some of these same screening tests could be adapted for use with tissue engineering products. Some of these organizations have discontinued their support of standards-writing activities.

5.4.1 US Pharmacopeia

The United States Pharmacopeia (USP; www.usp.org/aboutUSP/) is "a non-governmental, official public standards-setting authority for prescription and over-the-counter medicines and other healthcare products manufactured or sold in the United States. USP sets standards for the quality, purity, strength, and consistency of these products – critical to the public health. USP's standards are recognized and used in more than 130 countries around the globe."

In 1967 the USP adopted an implantation protocol first proposed by Lawrence *et al*. (1963), as part of their classification scheme for plastic containers. While most chronic tests include implantation times of longer than 30 days, the USP implantation protocol has a 3-day minimum duration. The standard protocol published by the USP includes a description of the specimen size and shape and implantation and evaluation procedure. This protocol (along with the other USP *in vitro* procedures) is a frequently used test for screening candidate implant materials.

5.4.2 American Dental Association

In 1972 the American Dental Association (ADA) Council on Dental Materials and Devices published standardized laboratory procedures for screening materials and devices (Council on Dental Materials and Devices, 1972). The stand-

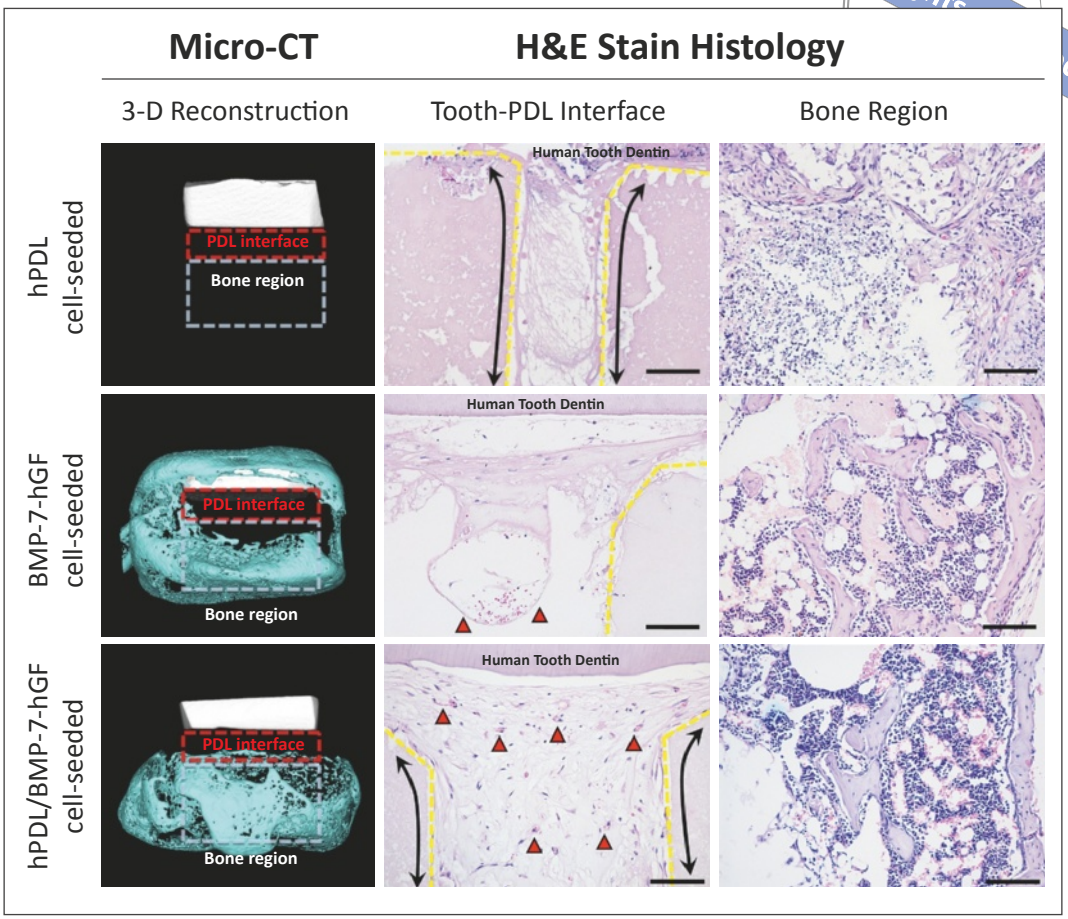

Fig 5-5 Tissue-engineered tooth-ligament-bone constructs. Three-dimensional reconstructed colorized micro-CT images and hematoxylin and eosin (H&E) stained histologic slices of tooth-ligament-bone constructs. The mineralized tissue (blue) was formed around the hybrid scaffolds and there was no ankylosis or bone fusion to the dentin surface (white). Red and blue dashed-line boxes were represented with periodontal ligament (PDL) interface and bone region, respectively. H&E stained histologic slices show the PDL interface and bone region tissues to evaluate fibrous tissue orientation along the column-like structures in the PDL interface, which were designed with a perpendicular direction to the dentin surface. The yellow dashed line is the borderline of channel-type PDL architecture and the black-arrowed lines represent the fibrous cell/tissue directionality following the wall of PDL interface structure. Red triangles indicate the blood vessels and yellow triangles point to the cementum-like tissue layer or cell deposition for cementogenesis on the dentin surface. Scale bar: 125 mm. Reprinted from Park *et al*. (2010), with permission from Elsevier.

ard protocols included short- and long-term implantation tests. Chronic implantation protocols included in the ADA-approved standards utilized subcutaneous as well as osseous tissue as the site of implantation. The standards provided details of the specimen preparation and implantation and evaluation procedure. While the test protocol is not often employed currently, it is an example of the type of standardized test that can be employed for a wide range

of biomaterials. In the subcutaneous protocol that was initially adopted, test materials were placed into 10 mm long Teflon tubes having an inside diameter of 1.3 mm. Tubes were inserted into pockets made in the subcutaneous tissue of each of the four dorsal quadrants of guinea pigs. Short-term tests had a 2-week duration, while the long-term tests are terminated after 12 weeks. At least four specimens at each time period needed to be evaluated histologically. The tissue reaction to the test material as viewed at the ends of the Teflon cylinders is compared with the "control reaction" adjacent to the midsection of the tube. The tissue reaction at the 2- and 12-week periods was classified as:

- No to minimal tissue reaction
- Moderate tissue reaction
- Severe tissue reaction.

The classification was based on the degree of inflammation and types of cells present. Materials which caused no-to-slight reactions were considered acceptable for usage tests in humans, those which elicit moderate reactions need further testing to establish the irritant components before usage tests, and those which cause severe reactions are considered unacceptable. In the standard recommended practice for implantation in bone, test specimens were placed into cylindrical Teflon cups. The cups were open at one end and had an outer surface containing a spiral ridge or treads. The cylinder was 2 mm long with an inner diameter of 1.3 mm and an outer diameter of 2 mm. Holes were drilled in the distal ventral symphyseal region of the mandibles of guinea pigs. One test cup was implanted in each half of the mandible with the open end toward the spongy bone. The periods of evaluation were 4 and 26 weeks. Forty specimens were to be evaluated at each time period. At necropsy, segments of bone including the test cups were decalcified and embedded in paraffin. The interface area at the opening of the cup between the test material and bone was to be evaluated for:

- Presence or absence of necrosis and inflammation
- The intensity of inflammation
- Resorption and possible replacement of the test material
- Presence or absence of bone, osteolytic, osteosclerotic, or osteoclastic activity.

The protocol outlined the criteria to be used in classifying the reaction as mild, moderate, or severe. The ADA-approved standards also included (Laing *et al*., 1967) tests to determine the mucous membrane and pulp irritation of materials.

5.4.3 American Society for Testing and Materials (ASTM)

The ASTM is a voluntary standards-writing organization which develops and approves standards by consensus. In 1972 the society approved a standard recommended practice for experimental testing for biological compatibility of metals for surgical implants. The standard was developed by the F4 subcommittee on surgical implants. The chronic implantation protocol described in the standard was essentially derived from some of the earlier investigations conducted by Laing *et al*. (1967). Cylindrical test specimens of metal are implanted in muscle or bone and the tissue reaction compared with control specimens implanted in the same animals.

5.4.4 International Standards Organization (ISO)

The ISO includes about 80 member bodies, each representing a national standards institute or bureau. In 1972, a technical committee (TC 150) was founded to consider standards relating to surgical implants. Constituent subcommittees and working groups have subsequently developed standards for a wide variety of implant materials and devices.

5.5 Summary

The screening of tissue engineering products is an essential step in the assessment of the potential utility of the constructs. There is an array of standardized test protocols employing bone and soft tissue implant sites which can be performed before the products are evaluated in models to determine their efficacy.

Acknowledgment

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