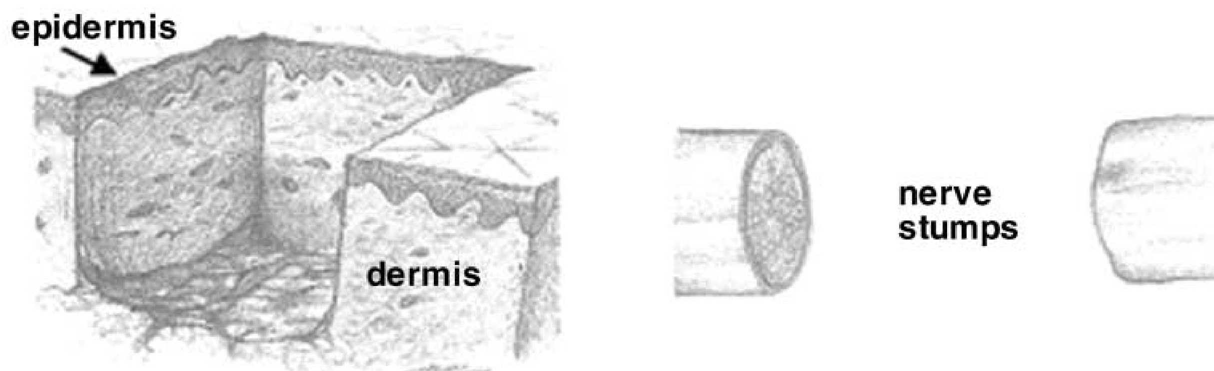
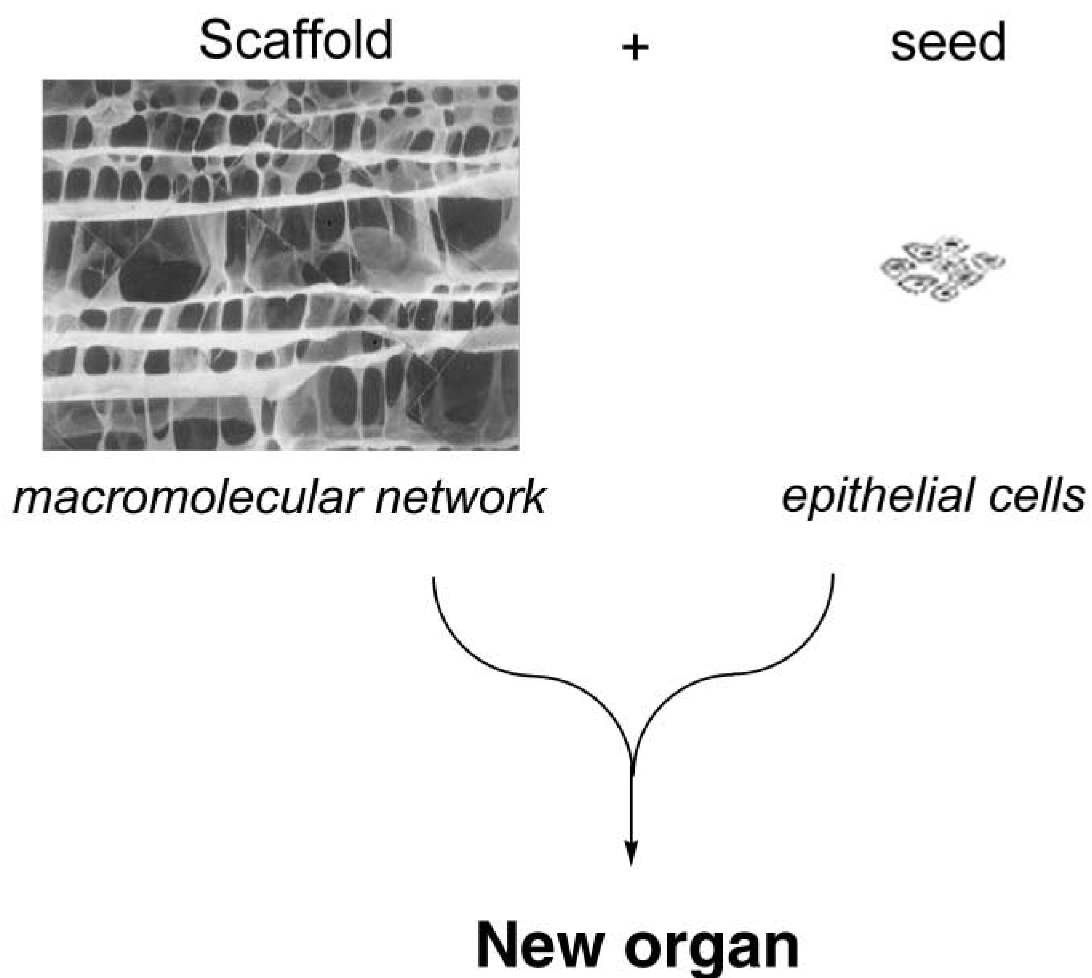
Tissue damage



Regeneration process



New organ

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Synthesis of Tissues and Organs

Ioannis V. Yannas*[a]

Symbolism that describes the synthetic processes for chemical compounds has been used to describe, in qualitative terms, the synthesis of tissues and organs at the correct anatomical site. The synthetic process is summarized in the reaction diagram, a shorthand representation of the reactants, reactor, and products. Analysis of a large number of independent protocols has led to identification of the simplest synthetic pathways for two organs that have been studied extensively: skin and peripheral nerves. These apparently irreducible reaction diagrams for the two organs are not only simple but surprisingly similar, a fact suggesting the existence of general rules for the synthesis of other organs as well. The only two reactants that are required are an active scaffold (a macromolecular network synthesized as a highly porous analogue of the extracellular matrix) and a seeding of epithelial cells of the organ being synthesized. Scaffolds possessed biological (regenerative) activity provided that they were capable of blocking the contraction process that leads to closure of the injured site. Such activity requires that the density of ligands for binding of contractile cells on the scaffold maintains a sufficiently high level over the period of synthesis.

Introduction

The synthesis of tissues and organs is having an increasing impact in the clinical setting, in assisting patients who have irreversibly lost the use of an organ. Although conventional approaches for organ replacement, such as transplantation, autografting, and implantation of engineered prostheses, are extensively used, the process by which a patient can regrow (regenerate) a lost organ at the correct anatomical site is, in principle, undoubtedly more attractive. A regenerated organ is a genuine part of the anatomy and does not suffer from problems of biological incompatibility; it even grows as a child host grows. In spite of the fact that the syntheses achieved in this developing field have so far been partial, the clinical benefit is remarkable.

One of the major problems in this field is a lack of understanding of the basic rules that govern such synthetic processes. This has led to a proliferation of processes for the synthesis of nearly identical tissues and organs. Careful inspection of the lengthy, complicated protocols that are employed provides very little insight into what is required and what is redundant. There is an urgent need for generalization and simplification.

Even the most involved synthetic pathways that have led to the synthesis of organic compounds pale in complexity compared to those employed for the synthesis of biological organs. This discrepancy makes one wonder about the value of chemical symbolism in this biological context. After all, how can a cell be represented simply as a reactant? How useful is it to represent an anatomical site as a reactor? Can an absence of information during the synthesis about the space- and time-dependent concentration of several cytokines, admittedly critical reactants, be neglected? And so on.

All these questions are justified. The answers hinge directly on the objective that is pursued when the symbolism developed for use in one science is used in another. Chemical symbolism is used here almost exclusively in order to identify the simplest ("irreducible") synthetic pathway, the one in which each of the reactants employed is necessary (required) to produce the desired organ. Once identified, this pathway not only simplifies immeasurably the experimental design but also immediately sharpens the focus for hypotheses about the mechanism of the synthetic process. Furthermore, comparison of the simplest pathways for synthesis of two different organs, skin and peripheral nerves, can be made quite directly and similarities can be deduced.

In fact, a surprising result has been observed following this analysis: In spite of dramatic differences in structure and function between skin and peripheral nerves, the reactants that are required to synthesize them, though not identical, are anatomically similar. Can such a similarity suggest approaches to the synthesis of other organs as well?

This article identifies the rules that lead to highly simplified protocols for organ synthesis and describes certain implications and uses of these rules. A brief mechanistic interpretation of the empirical rules follows. A detailed analysis of the data that support these rules, as well as of the mechanistic interpretation of the data, has been presented.[1]

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Symbolism for Reactants, Reactors, and Products

We seek a method for a comparative analysis of protocols that have been used by a large number of independent investigators, mostly biologists, physicians, chemists, and engineers, to synthesize tissues and organs. The objective of the analysis is identification of the simplest conditions for a given synthesis.

How much information is required to describe a protocol in sufficient detail to allow comparison of widely different approaches? These are biological processes in which cells synthesize tissues and organs while their activity is regulated by soluble macromolecules (e.g., growth factors, symbolically referred to here, for simplicity of presentation, as cytokines) and by insoluble networks (scaffolds). The sheer amount of information required appears, at first, to be unmanageably large. Recall, however, that our objective in this analysis is not a description of the absolute magnitudes that participate in a given reaction: It is rather to analyze the qualitative differences among processes used by investigators to synthesize a given tissue or organ. Rather than trying to focus on how many cells per unit volume or how many cytokine molecules per cell were used in a process (information not readily available), we wish instead to report on whether the cells used to synthesize a given tissue were, for example, fibroblasts or keratinocytes. We are attempting a purely qualitative analysis of processes; this is as far as the knowledge available to us today will let us go.

Tissue synthesis is carried out by cells that are regulated by certain cytokines and by matrices with specific structures. The reactants employed will, therefore, be classified into just three categories: cells, cytokines, and matrices. A review of a large number of protocols for tissue and organ synthesis published during the past 25 years has shown that investigators have added suspensions of cells of different types, solutions of one or more cytokines, and occasionally insoluble materials either into a cell-culture flask (in vitro reactor) or into an anatomical site of a living organism (in vivo reactor).

Specification of the cell type is obviously justified since, for example, the fibroblasts from a given organ typically synthesize quite different proteins to the epithelial cells of the same organ. Although there is evidence that the number of times cultured cells have been passaged prior to being introduced into the reactor affects the identity and quantity of proteins that they synthesize, there are few reports of the state of cell differentiation in investigations that have led to synthesis of tissues and organs. Information about passaging of cells used as reactants will be omitted in this analysis. For further simplicity, information about the composition of the culture medium used in vitro will also be omitted. Chemically defined media, constituted from chemical compounds at standardized concentrations, have been increasingly used in vitro for the synthesis of skin tissues[2] or peripheral nerve tissues,[3] thereby minimizing greatly the large differences in composition of media that were occasionally observed in earlier studies.

Solutions of cytokines are specifically cited as reactants in our analysis provided that they were introduced by the investigators (exogenous cytokines). In contrast, explicit mention of cytokines that are supplied by the tissue exudate that flows into the wounded anatomical site following surgical preparation is omitted. Such an omission is glaring, when one considers the high concentration of several cytokines in the typical exudate and the strong regulatory effect that certain cytokines have on cells. The omission is justified on two counts. First, we currently have little detailed information on the time- and space-dependent concentration changes of the several cytokines that play important roles during synthesis. We will refer to this information as being descriptive of a "cytokine field". Although the identity of most cytokines that regulate cell behavior in many injured anatomical sites has been established, the details of the conversation among cells (cell–cell signaling), for which these soluble regulators form the vocabulary, are largely unknown. Second, it can be argued that, provided the wounded anatomical sites prepared by two independent investigators have been prepared by an identical surgical protocol, as stipulated immediately below, the exudate flowing inside the wound immediately after injury is identical, or nearly identical, in composition as well ("uniform" across different investigators) and so is the initial cytokine field. It follows that omission of the cytokine field from a qualitative description of the synthetic events that proceed at standardized wounded anatomical sites, all of which are expected to have the same cytokine field, does

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not handicap the comparative analysis that we will generate. This omission is referred to as the approximation of the "uniform cytokine field". The approximation currently appears to be not much more serious than the frequent omission in the literature of a specification of one of the standardized background variables, for example, the standardized pH value or temperature, from the protocol. Even so, however, the approximation is expected to apply only during the initial conditions for the in vivo process, that is, immediately after the injury has been inflicted and before addition of any reactant. The approximation obviously applies only to in vivo processes. Detailed justification for omission of the cytokine field from the comparative description of in vivo processes has appeared elsewhere.[1]

Scaffolds that modulate cell function are nondiffusible (insoluble) regulators that function by making specific connections with cells (cell–matrix signaling). The connection involves a cell receptor (integrin) and an active site on the scaffold surface (ligand); several such receptor–ligand binding events usually take place simultaneously. Integrin–ligand interactions are highly specific and, as will be seen below, can have a dramatic effect on cell function related to synthetic events. Two examples of such active scaffolds appear in Figure 1. A detailed physicochemical description of the structural characteristics of scaffolds that have regenerative activity has been presented previously.[4]

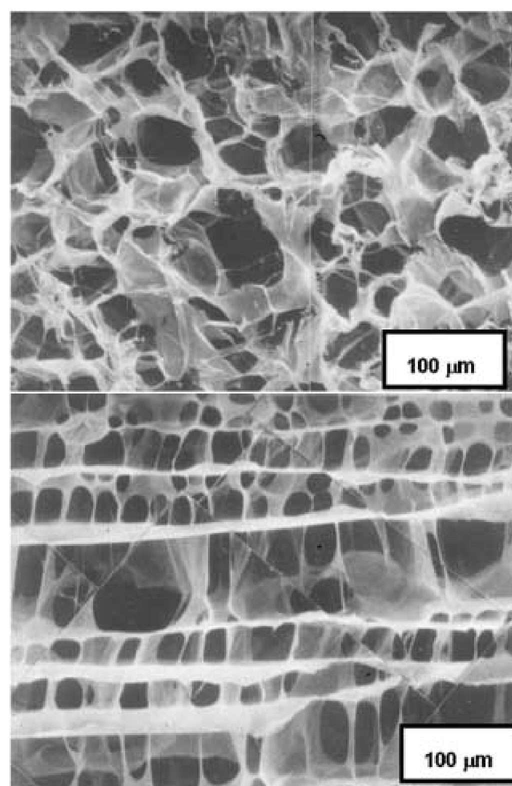

Figure 1. Scanning electron microscopy images of two scaffolds that have demonstrated high regenerative activity. The dermis regeneration template (top) is used as a graft to cover deep and extensive skin wounds; the nerve regeneration template (bottom) is used as filling for a nerve chamber in which the two nerve stumps of a transected nerve are inserted. Both were synthesized as highly porous graft copolymers of type I collagen and chondroitin 6-sulfate (a glycosaminoglycan or GAG). The structural properties of these scaffolds that need to be controlled within critical ranges are the chemical composition, the specific surface (increases with pore volume fraction and decrease in average pore diameter), the orientation of pore channel axes, and the degradation rate (decreases with the cross-link density). Bar: 100 μm. Courtesy of The Massachusetts Institute of Technology (M.I.T.).

The reactants described above are introduced into one of two basic types of reactor (Figure 2). In an in vitro protocol all reactants are introduced in the simplest biological reactor, a flask (typically located inside an incubator) that contains cell-culture medium. By contrast, an in vivo protocol takes place inside the living anatomical site where the organ is being synthesized. A suitable anatomical site is most simply prepared by a surgical procedure in which a mass of an organ is excised; the reactants are then implanted into that site. In studies with animals, the procedures involved have been described in great detail. They can be carried out readily by a skilled assistant.

Clearly, an in vivo reactor is much more complicated than its in vitro counterpart. For this reason, the majority of efforts to synthesize organs to date have focused on the simpler in vitro procedures. Careful study of in vivo reactors is, however, justified on the basis of two simple arguments. First, an organ that has been synthesized in vitro must eventually be implanted inside the anatomy of the host, that is, inside an in vivo reactor. Second, synthesis of clinically functional organs, even those that are only partially functional, has been demonstrated to date mostly with the products of in vivo reactors.

We now focus briefly on the advances recently made that simplify the interpretation of data obtained in such reactors. An important simplification is introduced by considering only processes that take place inside a small subset of standardized in vivo reactors. Use of standardized reactors drastically limits the variance in reactor conditions. The subset comprises special wounds, referred to as anatomically well-defined defects.[1] The space of such a defect consists of an experimental volume, free of tissues that do not regenerate spontaneously (nonregenerative tissues; see below); the volume is marked by unambiguous anatomical boundaries and it is physically constrained to prevent loss of exudate flow as well as to prevent entry of extraneous tissues or bacteria. Distinction among the different tissue types (epithelia, basement membrane, and stroma; see Figure 3) that must be deleted in order to generate the space of an in vivo reactor can be made on the basis of simple rules, described below.

Only data from two types of in vivo reactors have been considered below. We will consider in vivo reactors that have been used extensively by a large variety of investigators to study the two organs on which we will focus attention; they are the dermis-free skin defect (full-thickness skin loss) and the fully transected peripheral nerve (Figure 4). The protocols that have been included for analysis in this article have been carefully limited to the use of just these two reactors. Data from skin wounds of partial thickness or from nerves that were partially transected (for example, hemisectioned nerves) have been deliberately omitted. It has been shown that synthetic results obtained with surgical protocols that were only as different as the partial-thickness and full-thickness skin wound cannot be compared meaningfully to each other by using the information

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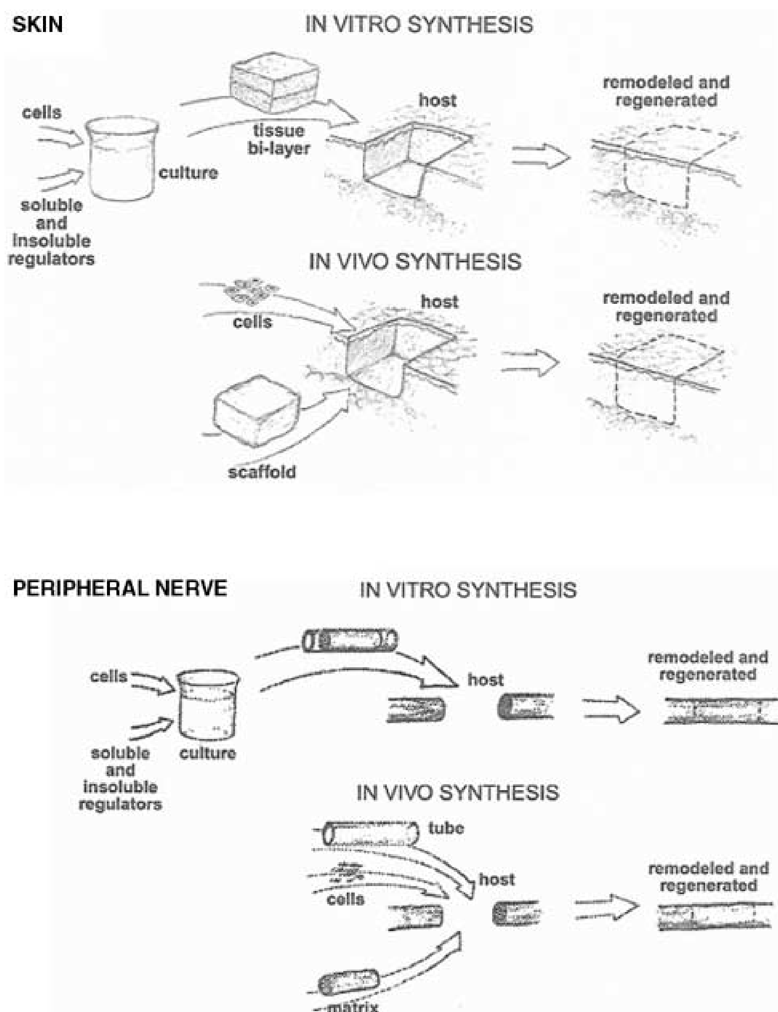

Figure 2. *Schematic representation of experimental configurations for in vitro and in vivo synthetic processes. Top: Skin. Bottom: Peripheral nerves. Reactants added by the investigator (exogenous reactants) include cell suspensions, cytokines (soluble regulators), and scaffolds (insoluble regulators). In a typical in vitro protocol reactants are first introduced into the culture medium; after several days, the resulting organoid is implanted at the correct anatomical site. In vivo protocols minimize the length of in vitro culture and typically proceed relatively directly to implantation of reactants at the anatomical site. (Reproduced from ref. [1].)*

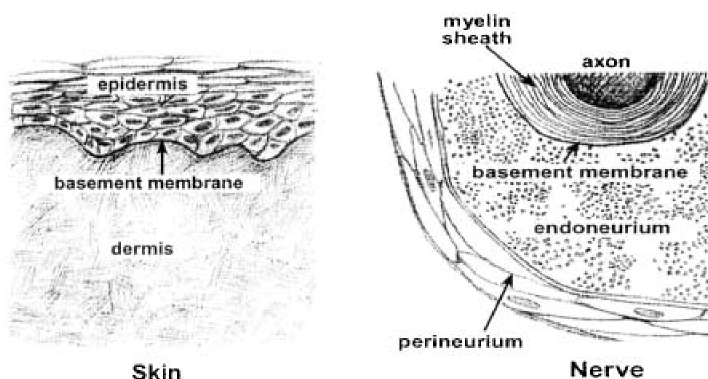

Figure 3. *The tissue triad in skin and peripheral nerves. The basement membrane, a very thin extracellular matrix (ECM), is flanked on one side by epithelia (epithelial tissue), a cellular and nonvascular tissue that lacks ECM; on the other side is stroma, supporting tissue comprising primarily ECM and blood vessels. In skin, epithelial tissue is the epidermis and stroma is the dermis. In nerves, epithelial tissue is the myelin sheath and stroma is the endoneurial stroma (endoneurium). (Reproduced from ref. [1].)*

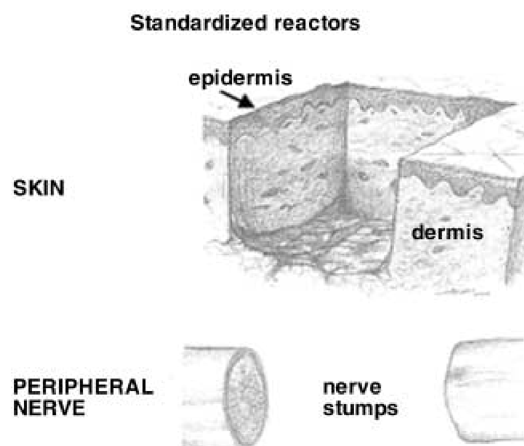

Figure 4. *Standardized reactors for in vivo synthesis of skin (above) and peripheral nerves (bottom). Data that lead to identification of the simplest synthetic pathways in this article derive exclusively from reports that were based on the use of just these two reactors. The reactor for the synthesis of skin and its tissue components is prepared by excision of the entire epidermis, basement membrane, and dermis (full-thickness skin wound). For studies on peripheral nerves, the reactor is prepared by complete transection of the nerve. Transection is typically followed by inserting the two nerve stumps inside a nerve chamber (tube) that may optionally contain a filling. (Reproduced from ref. [1].)*

that is currently available.[1] Inclusion of data obtained under conditions other than those shown in Figure 4 would have seriously confused the analytical procedure.

Although it appears at first that consideration of only data that have been obtained with a subset of reactors may have handicapped our analysis, the reverse is actually true. The substantial benefit we reap is the ability to conduct a direct comparison of the complex protocols used by independent investigators. This approach automatically excludes consideration of data that are not directly comparable from one protocol to another.

Products of the synthetic process are organs or simply their constituent tissues. A common classification of tissues in an organ divides them into members of the "tissue triad": Epithelial tissues are 100% cellular; they comprise no extracellular matrix (ECM) and have no blood vessels. The basement membrane comprises no cells and is made up entirely of ECM. The stroma contains cells, ECM, and blood vessels (Figure 3). As summarized in Table 1 for skin and peripheral nerves, adult epithelial tissue and the associated basement membrane are regenerative while the stroma is clearly nonregenerative.[1] This classification is a very useful method for product identification. (The same tissue classification was also useful in the design of the anatomically well-defined reactors shown in Figure 4.)

There is a dearth of quantitative assays for the identification of products, especially assays that distinguish between normal stroma and scar tissue. Since

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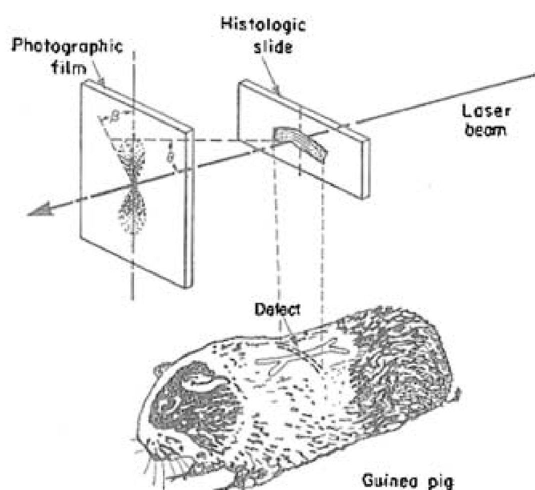
Table 1. Regeneratively similar tissues in skin and peripheral nerves (partial list).

	Skin	Peripheral nerves
Regenerative tissues	epidermis, basement membrane	myelin sheath, basement membrane
Nonregenerative tissues	dermis	endoneurial stroma

synthesis of the stroma occupies a central position in this field (see also below), the development of assays for scar tissue and for normal stroma is an essential step. A useful assay for the identification of scar tissue and its distinction from normal dermis is shown in Figure 5. Here, the light-scattering pattern from a conventional histologic slide is analyzed quantitatively to evaluate an orientation function that describes the orientation of collagen fibers in the tissue. The numerical value of that function is used to distinguish between scar tissue (highly oriented collagen fibers; linear scattering pattern) and dermis (quasi-randomly oriented fibers; elliptical pattern).[5]



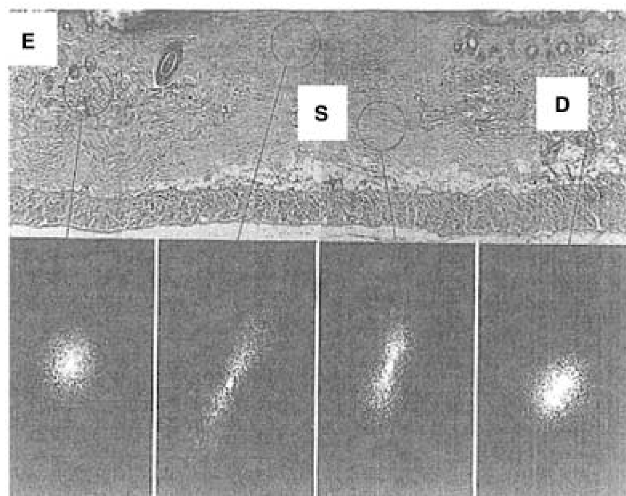


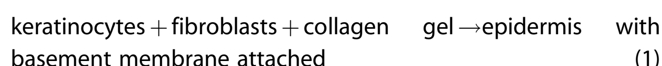
Figure 5. Quantitative distinction between scar tissue and physiological dermis can be made by using laser light scattering from tissue sections. Top: The laser beam passes through a histologic tissue section and is scattered. The scattering pattern is viewed on the photographic plane and is characterized by the scattering angle θ and the azimuthal (or rotation) angle β. The tissue section (scar) was sampled from a plane that was perpendicular to the long axis of the closed wound (full-thickness skin wound in guinea pigs that healed with scar formation). The major direction of contraction of the scar was perpendicular to the long axis of the closed defect. The data indicate that collagen fibers in scar tissue were oriented along the direction of the principal contractile stress. Bottom: Four areas of the tissue section were sampled by the light beam. The scattering patterns (below the microscopy section) show that the collagen fibers in scar tissue are oriented in the plane (quasilinear patterns inside left and right) while fibers in normal dermis are quasirandomly oriented (elliptical patterns, outside left and right). E = epidermis, D = dermis, S = scar tissue. (Adapted from ref. [5]).

Shorthand notation is employed to describe product tissues and organs; for example, in skin, the epidermis is abbreviated as E, basement membrane is BM, rete ridges (undulations in the dermal–epidermal junction characteristic of skin but not of scar tissue) are RR, and the dermis is D. The anatomical connection between two adjacent tissues is represented by a dot between symbols for neighbors; for example, partially synthesized skin is represented as E•BM•RR•D. All abbreviations, including those used for tissues in peripheral nerves, are listed in a footnote of Table 2.

Reaction Diagrams: Uses and Limitations

Information about the protocol used in a synthetic process is concisely summarized in the reaction diagram, a qualitative shorthand description of the reactants introduced in the reactor and the products that resulted. All reactants explicitly referred to in a reaction diagram are only those that have been added by the investigators into the reactor (exogenous reactants); endogenous reactants, whether provided by the culture medium (in vitro) or the flowing exudate (in vivo), are omitted. (Explicit information on the reactor used is provided only with an in vivo process and is designated by appropriate notation on top of the arrow that shows the direction of the process.)

An example of a reaction diagram is shown below,[6] written in longhand [Eq. (1)] and then in abbreviated form [Eq. (2)]:



$$\text{keratinocytes} + \text{fibroblasts} + \text{collagen gel} \rightarrow \text{epidermis with basement membrane attached} \quad (1)$$

$$\text{KC} + \text{FB} + \text{COG} \rightarrow \text{E}\cdot\text{BM} \quad (2)$$

This reaction diagram informs us that keratinocytes (KC), fibroblasts (FB), and a collagen gel (COG) were added to an in vitro reactor, resulting in the synthesis of a fragment of an organ, an epidermis with the associated basement membrane attached to it (E•BM). This diagram is a shorthand version of detailed protocols published independently by several teams of investigators over a 10-year period.[6–12] (Although a protocol for the synthesis of E•BM was published in each case, the major objective of some of these investigations was not the synthesis of E•BM.)

The reaction diagram shown above is an example; it does not describe the simplest conditions required to synthesize the organ fragment E•BM. However, inspection of several reaction

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diagrams from different investigators, all of whom synthesized E•BM, can rapidly lead to identification of the irreducible reaction diagram, that is, the process for synthesizing tissue that requires the minimum number of reactants or that takes place under simpler conditions (in vitro considered simpler than in vivo). The simplest diagram for the synthesis of E•BM turns out to be that shown in Equation (3):[3]

$$\mathrm{KC} \rightarrow \mathrm{E \cdot BM} \quad (3)$$

We conclude that neither fibroblasts nor any scaffold gel were required to achieve an in vitro synthesis of E•BM.

The simplest (irreducible) reaction diagram shows at a glance the minimal requirements for a given synthesis. Clearly, such a diagram is not immune to being replaced in future studies by an even simpler diagram. It only answers the question: Based on the available data, which reactants, added to which reactor, are required (necessary) in order to synthesize the tissue or organ of interest? In a field that is noted for its highly complex protocols, often extending over a few pages, the economy introduced by such a diagram is not to be slighted. Furthermore, once identified, the simplest process cannot be neglected in future studies of the mechanism of synthesis.

The limitations of this shorthand presentation are the result of the approximations employed in setting up and interpreting the reaction diagrams. One such approximation is the use of a single name for a product, irrespective of its degree of maturation (differentiation). This problem is dealt with by accepting as valid the report of a synthetic event provided that the investigator has identified the product by use of at least one recognized assay. Clearly, this criterion is arbitrary. Since the identification of tissues and organs is not yet as precise as that of a chemical compound such as (say) nitrobenzene, there is an obvious opportunity for some ambiguity. Another limitation is introduced by the assumption that an individual tissue or organ fragment can be synthesized in an "out-of-organ context", that is, as a discrete, stable entity such as E or E•BM, without making contact with the tissues to which it is normally connected inside the organ. A simplified view of an organ as a "linear assembly" of constituent tissues, in which individual tissues exist outside the organ setting, has been occasionally supported by independent evidence.[14, 15] Tissue synthesized in an out-of-organ context is typically nonvascularized and is frequently unsupported metabolically by the organism; eventually, such a tissue shows signs of instability. However, eventual loss of stability does not affect the identification of freshly synthesized tissues, just as the inherent instability of a reactive intermediate or free radical in a chemical reaction does not invalidate methods for the identification of these transient species.

Further ambiguity is introduced by the omission of explicit information on the cytokine field; this is the uniform cytokine field approximation (see above). It is based on the assertion that the cytokine field does not vary among investigators (or among animals within the same protocol) provided that the anatomical site of synthesis is maintained as fixed in the different protocols. What is the evidence that this approximation holds true when data from the nominally identical reactors have been obtained with different species? This question cannot be answered today because the available information on cytokine concentration levels in different reactors is so limited. Finally, the reaction diagram contains no stoichiometric information and is clearly not a chemical equation. Also, like a chemical equation, it contains no information on the kinetics of the synthetic process.

Simplest Reaction Pathways for Skin and Peripheral Nerves

Detailed reaction diagrams describing several protocols from different investigators who synthesized either skin or peripheral nerves have been presented in detail elsewhere.[1] These diagrams describe 33 protocols for the synthesis of tissue components of skin (epidermis, basement membrane) and partial skin itself, as well as 20 protocols for the synthesis of peripheral nerve tissue components (myelin sheath, basement membrane, endoneurium, perineurium) and the conducting nerve trunk (20 additional protocols). In this article we omit discussion of the synthesis of the perineurium; we also omit the complete listing of the numerous protocols for skin and nerve synthesis.[1] We describe below the outcome of this analysis.

The simplest synthetic pathways deduced from these protocols are shown in Table 2. Synthesis of the epithelial tissue in skin (epidermis, E) requires in vitro culturing of keratinocytes but no other cell type, cytokine, or scaffold.[2, 16, 17] A slightly different in vitro protocol that also makes use of only keratinocytes has been shown to yield epidermis with its associated basement membrane, E•BM.[13] However, synthesis of the dermis, D, requires in vivo conditions with the use of an active scaffold such as the dermis regeneration template (DRT), an insoluble analogue of the extracellular matrix.[18–20] Likewise, synthesis of the skin organ has not been observed in vitro; the in vivo protocol yields partial skin, E•BM•RR•D, that is, skin with epidermis, basement membrane, rete ridges, and a dermis with blood vessels, nerves and mechanical strength but no appendages (hair, sweat

Table 2. Simplest synthetic pathways[a] for epithelia, basement membrane, stroma and the partial organs, skin and peripheral nerves.

	Epithelia (in vitro)	BM (in vitro)	Stroma (in vivo)	Organ (in vivo)
skin	KC → E	KC → E•BM	DRT → D	KC + DRT → PS
nerve	SC → MAX	SC → MAX•BM	tube → ED(?)	SC + tube → nerve trunk

[a] Abbreviations for skin: KC = keratinocytes, E = epidermis, BM = basement membrane, E•BM = tissue comprising epidermis with BM attached; DRT = dermis regeneration template, D = dermis, PS = partial skin (no appendages). Abbreviations for peripheral nerves: SC = Schwann cells, MAX = myelinated axon, MAX•BM = myelinated axon with BM attached, tube = nerve chamber with regenerative activity, ED(?) = endoneurial stroma (partial evidence for synthesis), nerve trunk = regenerated nerve that conducts electrical signals.

glands). Partial skin synthesis in guinea pigs and swine requires the use of DRT that has been seeded with keratinocytes.[18–27] An apparently complete in vivo synthesis of the basement membrane region (dermal – epidermal junction) in swine by the use of only the keratinocyte-seeded DRT has been documented; the presence of rete ridges with capillary loops is evidence of the lack of scar synthesis (Figure 6).[24]

The partial synthesis of skin discussed above takes place by simultaneous, rather than sequential, synthesis of its tissue components: All reactants are added at the same time. Alternatively, the tissue components comprising the entire organ can conceivably be synthesized sequentially in vivo, that is, the dermis is first synthesized by using the DRT and this is followed by spontaneous regeneration of the epithelia and basement membrane on the presynthesized dermis.[1] Although this sequential synthetic route requires fewer exogenous reactants (keratinocytes are supplied not by the investigator but by the anatomical site) it leads to a greatly delayed synthesis of skin,[28] a result that is normally undesirable in a clinical setting.[29] However, it is an experimental alternative that can be used to improve understanding of organ synthesis. In the clinical setting where the use of exogenous cells, even when autologous, is complicated, an autoepidermal skin graft (removed from an intact area of the patient) is used to seal off the dermis that has been previously regenerated in the injured site simply by use of DRT grafting.[30–32]

Synthesis of peripheral nerves requires protocols that depend heavily on the use of Schwann cells and an active scaffold. Schwann cells, the epithelial tissue component of peripheral nerves,[33] are the only reactant required to synthesize myelinated axons (MAX) as well as complete nerve fibers comprising myelinated axons with their associated basement membranes (MAX · BM).[3, 34–37] With one exception,[38] synthesis of the endoneurial stroma does not appear to have been explicitly reported (probably because the vast majority of investigators have focused instead on assays for the synthesis of axons). However, synthesis of a conducting nerve trunk, with indirect evidence of the presence of some kind of endoneurial stroma, has been

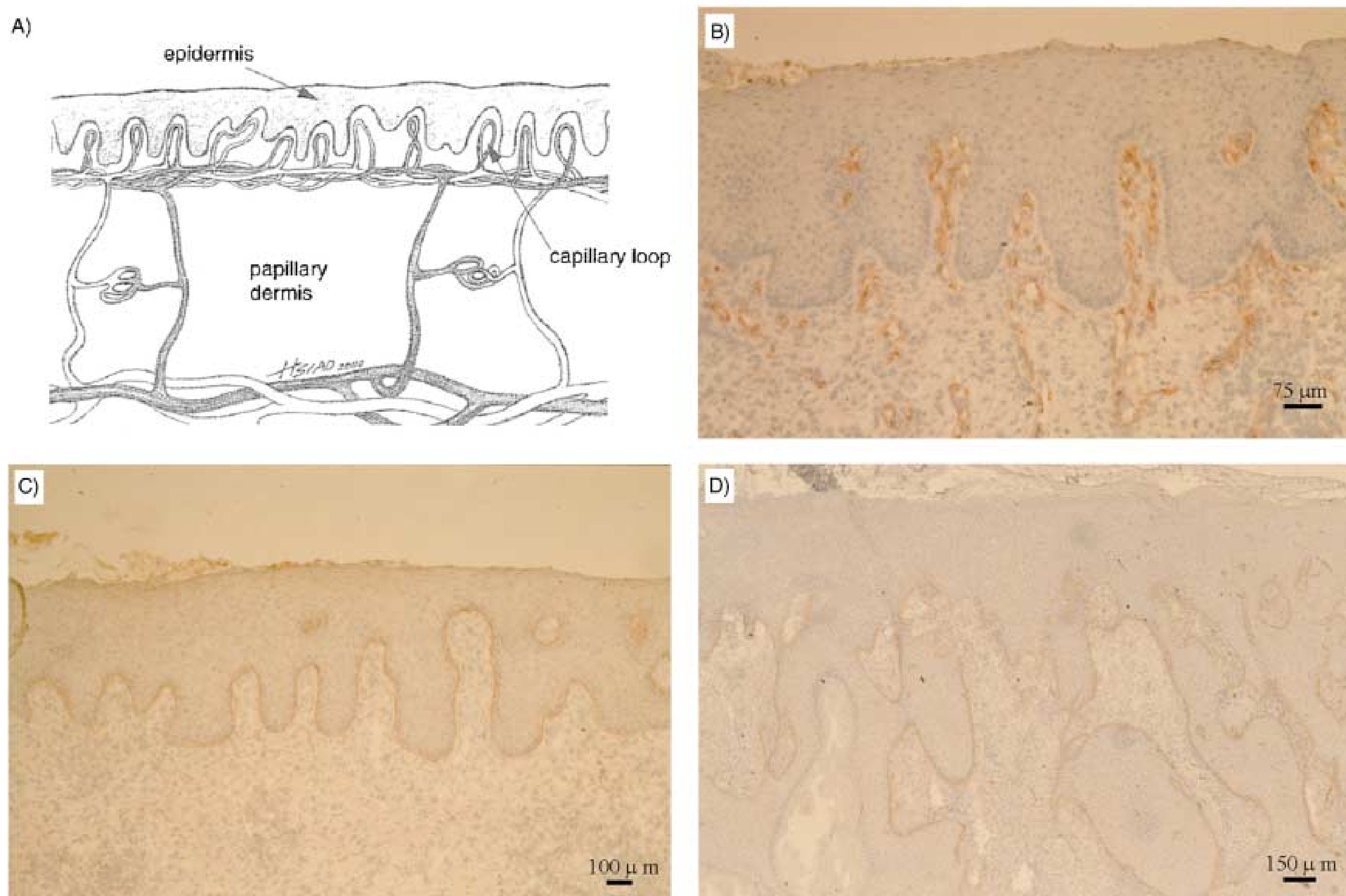

Figure 6. Evidence of a normal dermal – epidermal junction following synthesis of skin by grafting with keratinocyte-seeded DRT. A) Normal skin. Schematic representation of vascular loops (plexus) interdigitated with undulations of dermal – epidermal junction (rete ridges). B) Regenerated skin. Vascular loops have been synthesized in the rete ridges; they are identified by immunostaining with Factor VIII 35 days after grafting. Notice the similarity to the sketch of the normal plexus (A). C) Skin regenerated as above. Hemidesmosomal junctions, connecting basal cells to dermal structures, have formed after 35 days, as shown by immunostaining for $\alpha_6\beta_4$ integrin, the cell receptor that binds to dermal ligands. D) Skin regenerated as above. Anchoring fibrils, connecting epidermal basal cells (most proximal epidermal cells) to the dermal matrix, were labeled by immunostaining for type VII collagen. These fibrils formed early in the synthesis, at 12 days, before the newly synthesized epidermis had separated from the dermis. (Reproduced from ref. [24].)

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reported on a very large number of occasions.[1] Somewhat unexpectedly, the tube (nerve chamber) that is universally employed in protocols that lead to the synthesis of a conducting nerve trunk has been shown to play much more than just the physical role of guiding regeneration: There is now considerable evidence that the physicochemical structure of the tube may, if properly selected, have a profound regenerative activity of its own, a biological activity independent of its often-cited role as a "physical guide". Such activity resides in the tube wall and is separate from any activity potentially residing in the filling that is occasionally placed inside the tube.[1, 40, 41]

The field of tissue and organ regeneration is still in an early stage of development; the organs synthesized to date are not fully physiological. Although the regenerated skin has nerves, blood vessels, and mechanical strength, it lacks skin appendages (hair and seat glands).[18–25, 30, 42, 43] Peripheral nerves, synthesized in the standard rat sciatic nerve model across a gap of 10 mm, conduct electrical signals at about 75% of normal velocity; however, the strength (amplitude) of the signals is only about 30% of the normal value.[40] Even though medical devices developed on the basis of these regenerated organs are in relatively wide use (see below), the deficiencies provide challenges for the future.

Kinetics of Organ Synthesis

The kinetics of skin synthesis are illustrated in Figure 7. We focus first on the synthesis of the epidermis and the associated basement membrane.[24, 27] The reactants used in this synthesis were the keratinocyte-seeded dermis regeneration template (DRT). The epidermis is synthesized first and separates itself from the newly synthesized dermis (in a process that resembles cell-segregation phenomena observed in vitro; Figure 7). Keratinocytes are observed inside the scaffold as isolated cells by day 4. By day 8, keratinocytes have started to condense into islands and cords; a segmented basement membrane has been also formed. Partial epidermal confluence occurs by day 12 and the basement membrane is continuous by that time. Keratin cysts (of unclear physiological significance) form by day 15 and are extruded out of the preparation by day 19. Maturation of the epidermis (keratinization) is virtually complete after 35 days.

Synthesis of the dermis generally takes longer than that of the epidermis. By day 4 the DRT is populated by cells, including mononuclear cells, granulocytes, red blood cells, multinucleated giant cells, and cells shaped as fibroblasts. Cell adhesion on the DRT fibers is observed by day 8. Synthesis of blood vessels (angiogenesis) and synthesis of stroma proceed by day 12 while, simultaneously, the DRT is undergoing extensive degradation. Random alignment of axes of fibroblasts is observed by day 15 and synthesis of the dermis is intensified by day 19. Rete ridges are synthesized by day 35.[23, 24]

A brief account of the kinetics of peripheral nerve regeneration inside a nerve chamber starts with the regeneration of nerve fibers. This account will focus on data obtained with the use of silicone tubes; the majority of kinetic data have been observed with such tubes. By day 7, a fibrin cable forms inside the nerve chamber; it is immersed in exudate and it connects the two stumps across the gap. The cable comprises primarily longitudinally oriented fibrin fibers; fibronectin is also present. At day 14, surface blood vessels are observed on the surface of the cable. Schwann cells and fibroblasts advance from both stumps by day 14, clearly ahead of nonmyelinated axons. After 23–25 days, the 10-mm gap is being traversed by nonmyelinating axons that elongate from the proximal stump. Myelinated axons appear with a delay of 7–9 days.[44] The thickness of the myelin sheath is not uniform along the length of the gap; it is thicker near the proximal end, a fact suggesting an ongoing

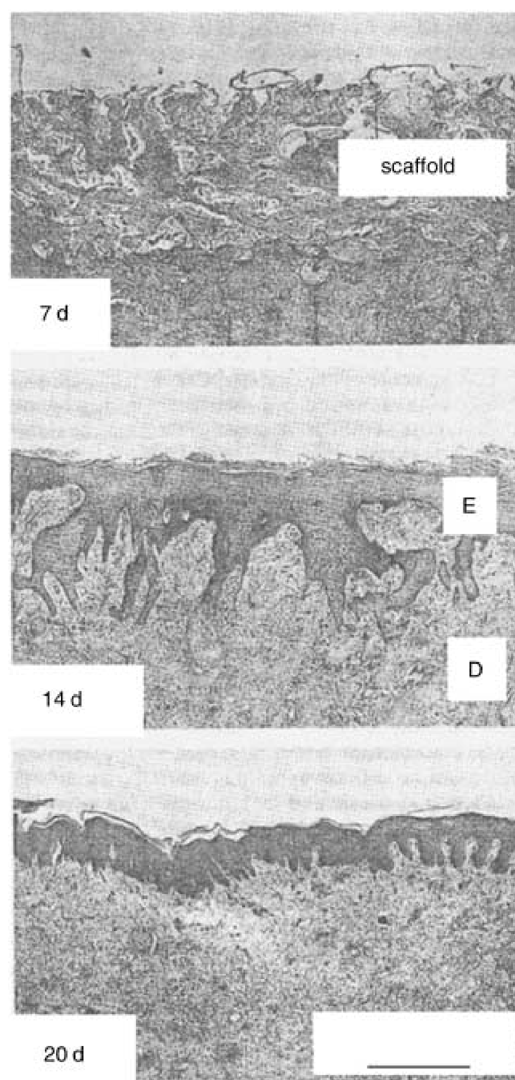

Figure 7. The kinetics of skin synthesis are illustrated by a series of cross-sectional views of the dermal–epidermal junction at 7, 14, and 20 days after grafting. A dermis-free defect (a standardized reactor) was grafted with the keratinocyte-seeded DRT. In this study, a combination of in vitro and in vivo methodology was utilized; prior to grafting, keratinocytes from the host (autologous) were cultured for 14 days before seeding into the porous DRT. Epithelial islands and cords surrounded by a discontinuous basement membrane (BM) formed by day 8; the BM became continuous by day 12. Epithelial islands and cords eventually condensed to form a fully confluent epidermis with a continuous basement membrane by day 19. Blood vessels had appeared in the forming dermis after 12 days, synthesis of collagen fibers in a quasirandom array was observed by day 19, and rete ridges were synthesized by day 35. Bar: 1 mm. (Reproduced from ref. [27].)

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axon maturation process. Myelination is well advanced near the proximal end before any axons reach the distal side of the cable. The total number of myelinated axons in the proximal nerve stump rapidly increases to values above normal by day 100 and remains approximately constant for several weeks thereafter.[40] The average diameter of a regenerated axon is clearly smaller than normal; however, there is a steady increase in average axon diameter that continues even at day 400.[40]

The kinetics of synthesis of the stroma depend strongly on the identity of material used to construct the nerve chamber. When a silicone tube is used, thick concentric layers of elongated cells and connective tissue form around the circumference of the early regenerate inside the silicone tube by day 14. Very little connective tissue appears to form around the regenerate formed inside a collagen tube. Use of immunostaining for α-actin shows these elongated cells to be myofibroblasts (contractile fibroblasts).[41] Synthesis of endoneurial collagen fibrils is first observed after 14 – 18 days. Cylindrical bundles of axons, smaller than in a normal nerve trunk (minifacicles), form by day 16. By day 21, blood vessels are observed along the entire length of the regenerated nerve inside the chamber. Synthesis of the perineurium is observed to proceed between day 16 and about day 180. The average diameter of the axons increases continuously, even as late as about day 400; concomitantly, the fraction of cross-sectional area of the nerve trunk that is occupied by nerve fibers also increases. Since the total cross-sectional area of the nerve trunk does not increase during this process, these changes suggest that extensive remodeling of the endoneurial tissue is taking place, probably leading to degradation of endoneurium and its replacement with remodeled tissue.[40]

Similarities between Synthetic Pathways for Skin and Peripheral Nerves

Both in skin and peripheral nerves, the epithelial component of the tissue triad for the organ (Figure 2) can be synthesized by culturing only cells that constitute epithelial tissue. These cells are keratinocytes and Schwann cells, respectively, for skin and peripheral nerves (see above for classification). There is no requirement for a cell of another type or for the presence of a scaffold. The basement membrane can also be synthesized in the same in vitro process without requirement for additional cell types or a scaffold. The stroma (dermis) can be synthesized in vivo following addition of a scaffold with appropriate regenerative activity. No dermal elements are required for the synthesis of the epidermis with its associated basement membrane, nor are any epithelial elements required for the synthesis of the dermis. Likewise, in studies of peripheral nerve synthesis, the myelin sheath with its basement membrane has been synthesized in vitro, in the presence of Schwann cells and the absence of elements of the endoneurial stroma. Furthermore, synthesis of a nerve trunk that functions in a partly physiological manner, for example, a trunk with the ability to conduct electrical signals, almost certainly implies synthesis of an endoneurial stroma. (Assays for identification of the latter have been largely absent in the literature.) Synthesis of a nerve trunk requires the presence of a nerve chamber (tube) that, if appropriately structured, functions as a scaffold of substantial regenerative activity.

It is remarkable that synthesis of neither skin nor peripheral nerves requires exogenous stromal cells (for example, fibroblasts). Nor is there a requirement for exogenous cytokines or for exogenous addition of cells that synthesize blood vessels (endothelial cells). Yet, the new skin has blood vessels, nerves, and mechanical strength; the new nerves also have blood vessels and conduct electrical signals. In both cases, the presence of physiological supporting (connective) tissue has been shown in the regenerated organs. It is conceivable that the synthesis would proceed to completion, with the synthesis of appendages in the case of skin and a more robust signal strength for synthesized nerves, if these components were included as reactants in an appropriate manner. There is also some evidence that addition of either of these as reactants may lead to acceleration of the kinetics for skin synthesis. Currently, synthesis of recognizable skin by using the required reactants, that is, the keratinocyte-seeded DRT (Table 2), takes about 20 days (Figure 7).

Use of these rules has been deliberately made during synthesis of the conjunctiva.[45] The approach used was the sequential synthesis of the conjunctival stroma and the associated epithelia by using only an active scaffold (DRT), as described above in the sequential synthesis of skin.

In summary, synthesis of the entire skin organ requires simply the combined addition of the two reactants that are necessary for the synthesis of the individual tissues of skin, that is, keratinocytes, for synthesis of the epithelia with the associated basement membrane, and the appropriate scaffold for synthesis of the stroma. The summary rules for the synthesis of peripheral nerves appear to be similar. Synthesis of a nerve fiber (myelin sheath with associated basement membrane) requires simply Schwann cells while synthesis of the nerve trunk (which most probably comprises newly synthesized endoneurial stroma as well) requires the presence of a tube acting as an active scaffold. This is a remarkably simple protocol for synthesis of an organ.

Modular Synthesis

Consideration of Table 2 shows that the entire skin organ, without appendages, and the entire nerve organ can be synthesized by using a protocol generated simply by “adding” the reaction diagrams for the synthesis of the two major tissue components. The data suggest that the whole (organ) can be synthesized directly as the sum of its parts (tissues).

The relative simplicity of these protocols suggests that almost the entire skin organ can hypothetically be synthesized in two spatially adjacent but distinct modules, each module designed to produce one of the tissue components. According to Table 2, the epithelia with the basement membrane could be synthesized in vitro while the stroma could be synthesized separately in vivo. In this example, the synthesis of each tissue component would take place independently of the other components in a separate experimental volume. At a later time, the products from each reactor would be brought into contact in order to synthesize the critical “transition” tissues, such as the dermal –

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epidermal junction in skin, including anchoring fibrils and rete ridges. The organ could then hypothetically be formed by suitable binding of the epithelia to the stroma.

Such a process of sequential synthesis, referred to as "modular organ synthesis", could be designed as a potentially simpler version of that in which all the reactants required to synthesize every tissue component of the organ are simultaneously fed into a single experimental volume, as currently practiced.

In Vitro or In Vivo Conditions for Synthesis?

It was concluded above, in discussions about both skin and nerve synthesis, that the epithelia with the associated basement membrane can be synthesized in vitro, while synthesis of the stroma requires in vivo conditions. Which indispensable reactants, apparently not available in vitro, are supplied in vivo? This question can be answered, at least in part, simply by considering the irreducible reaction diagrams that were selected in the preceding section (Table 2). A detailed analysis of the data appears elsewhere.[1]

Consideration of the data shows that an exogenous supply of fibroblasts is not required to synthesize any of the components of skin or peripheral nerves. Since it is well known that fibroblasts are critically involved in the synthesis of stroma,[46] and since stroma has been observed to have been synthesized, it follows that the fibroblasts must be supplied endogenously. A similar argument can be made about the absence of microvascular endothelial cells from the irreducible diagrams; these cells are responsible for angiogenesis.[47] Angiogenesis has been shown to have occurred in the regenerated organs without exogenous addition of endothelial cells.[23–25] We conclude, therefore, that synthesis of the stroma requires in vivo conditions partly due to the requirement for an endogenous supply of fibroblasts and endothelial cells.

If this simple analysis was sufficient to explain the data, synthesis of a vascularized dermis should be possible in vitro by seeding the hypothetically required fibroblasts and endothelial cells into an appropriate scaffold, such as the dermis regeneration template (DRT). Although studied independently, such an in vitro protocol has not led to the synthesis of vascularized stroma in vitro. Why? We recall that cell function depends both on cytokines and insoluble regulators (an appropriate matrix). Of these two classes of regulators, the appropriate cytokines, in the form of the cytokine field that is set up following an injury (see above), are clearly missing in vitro. Cytokines are present in the exudate that flows into the defect (cytokine field) very early on; they are also secreted by degranulating platelets and are further synthesized by cells migrating into the defect. Since there is no exudate nor platelets in a typical in vitro culture, the complex cytokine field is certainly missing in studies conducted in vitro.

In conclusion, the available evidence suggests strongly that the wound is a required supplier both of fibroblasts and endothelial cells, as well as of a cytokine field, during the synthesis of dermis. A similar conclusion is consistent with the available data from the synthesis of peripheral nerves. It is well known that cell function is regulated by cytokines during connective tissue synthesis (fibroplasia) and blood vessel formation (angiogenesis).[46–48] Clearly, fibroblasts, endothelial cells, and the cytokine field are related intimately and their functions cannot be considered separately. We conclude that it is not the separate requirement for the cytokine field nor for fibroblast or endothelial cell presence, but the specific regulation of fibroblast and endothelial cell function by the cytokine field, that must be primarily responsible for the uniqueness of the in vivo environment in the synthesis of certain skin components.

It is tempting to hypothesize that in vitro synthetic approaches cannot match in vivo studies unless the investigator duplicates in vitro the cytokine field that is spontaneously established in vivo following injury. Since information is not currently available about the cytokine field, this hypothetical in vitro approach would appear to have no advantage over in vivo synthesis. On the other hand, there is the occasional need to replace an organ without functional interruption over a period that does not exceed the time required to complete the surgical implantation procedure. For example, in the dynamic anatomical setting of a heart-valve replacement, implantation of a device that is designed to synthesize the defective heart valve in vivo but which requires several days to do so is not an attractive option. Unfortunately, our current knowledge of the cytokine field established following injury is not extensive. On balance, therefore, the state of the art currently favors in vivo synthetic routes.[49]

The Central Problem in Organ Synthesis

A detailed analysis of the irreversible response to injury in most adult organs makes it clear that the synthetic barrier to overcome during induced regeneration of most organs is the synthesis of the nonregenerative tissue, the stroma.[1] As shown by instances of sequential synthesis, the epithelia and the associated basement membrane are synthesized (regenerated) spontaneously following injury provided that the stroma has remained relatively intact (in minor injuries, such as a first-degree burn) or has been previously regenerated with an active scaffold. Another conclusion that emerges from the discussion above is that synthesis of the stroma requires an appropriately structured scaffold. It follows that the central problem in the synthesis of most organs is stroma synthesis, a process that requires the presence of an active scaffold. How does use of an active scaffold reverse the results of injury? In this section we summarize the mechanistic interpretation for this fundamental requirement. A detailed analysis of the available data that support this mechanism has been presented elsewhere.[1]

Following severe trauma of an adult organ in which the stroma has been severely injured, the wound closes by contraction and scar synthesis. When the stroma has been spared, the wound closes by spontaneous regeneration; contraction is not observed. A quantitative description of the processes by which wound closure takes place in severe wounds has suggested that contraction is the main engine for wound closure and that scar formation is a derivative process that depends critically on the presence of contraction. For example, in injured skin, scar tissue appears to involve synthesis of

connective tissue in the presence of plane stress; in injured nerves, scar tissue (neuroma) is formed apparently in the presence of circumferential stresses. Both in skin[50] and in nerves,[41] the stresses are generated by contractile cells (myofibroblasts). When contraction was blocked by the use of scaffolds in standardized skin wounds, even partially, scar formation was apparently absent, a result that suggests a requirement for the presence of a mechanical field in which contractile cells synthesize the highly oriented connective tissue (scar tissue). The apparently total inhibition of scar formation following even partial blocking of contraction suggests the secondary role of scar formation in the process of wound closure.

Certain analogues of the extracellular matrix, mostly graft copolymers of type I collagen and a glycosaminoglycan (GAG) such as chondroitin 6-sulfate (collagen – GAG copolymers), prepared in the form of highly porous networks (scaffolds), have shown a remarkable ability to inhibit contraction of standardized wounds when implanted at the site of the wound (Figure 1). The degree of inhibition of contraction has been related very closely to the structural characteristics of these copolymers. It has been shown that contraction is inhibited most actively when the physicochemical composition, the average pore diameter, and the degradation rate of the implant are controlled within relatively narrow ranges. The critical ranges of these structural variables are not identical in processes that lead to the synthesis of skin[21] or peripheral nerve.[51] Inhibition of contraction by an active scaffold has been shown to depend on successful completion of two processes: In the first, the active scaffold down-regulates the inflammatory response at the site of implantation, thereby reducing significantly the number of contractile cells. In the second process, the contractile cells are bound on to the extensive specific surface of the scaffold and lose their ability to contract the wound in a "coherent" manner.[1]

The significance of these phenomena becomes clear when it is recalled that scaffolds that have inhibited contraction of severely injured sites in skin and peripheral nerves have also induced partial regeneration of the injured organ. Such regenerative activity is remarkable because it has been rarely induced in adults; for example, no such activity has been exhibited when standardized wounds were treated with several types of solutions of cytokines, with cell suspensions, or with a large variety of scaffolds.[1]

A scaffold with regenerative activity in skin wounds (the dermis regeneration template) has been approved by the FDA as a device that is currently used to induce partial skin regeneration in patients with massive burns or those who have lost a significant mass of skin following reconstructive surgery. Another scaffold (the nerve regeneration template) has induced peripheral nerve regeneration across gaps of unprecedented length; an early version has been approved by the FDA for treatment of patients suffering from paralysis that resulted from severe injury in peripheral nerves of arms, legs, or the face.

There is strong evidence that inhibition of contraction is necessary but not sufficient to induce regeneration of the adult organs that have been studied. For example, inhibition of contraction following experimental treatment with steroids was not accompanied by regeneration; neither was regeneration observed following a significant delay of contraction in animal models of impaired healing, for example, mechanically splinted wounds, infected wounds, or wounds in genetically diabetic or obese animals.[1] Scaffolds with regenerative activity (regeneration templates) appear to inhibit contraction without blocking other processes involved in wound closure. Of these other processes, the most important in the context of induced regeneration is the synthesis of new physiological tissue. Considering induced regeneration as the sequence of two major steps, that is, blocking of contraction and synthesis of physiological tissue, we arrive at a useful conclusion: The central problem of organ synthesis in vivo is the identification of reactants that can block contraction without interfering with the synthesis of physiological tissues.

Regeneration templates have solved, at least in part, the central problem in organ synthesis by inhibiting contraction while inducing regeneration. The structural features that distinguish an active scaffold appear to be the identity of ligands for binding contractile cells (depends on the chemical composition), the ligand density (increases with specific surface, which is inversely related to the average pore diameter of the scaffold), and the duration of the implanted scaffold as an insoluble network (decreases with degradation rate; depends on chemical composition and network cross-link density). These properties can be controlled to appropriate levels by well-known physicochemical processes that have been described in detail.[4, 52, 53] Control of scaffold structure is critical. For example, the skin-regenerative activity of a series of collagen – GAG scaffolds peaked when the average pore diameter was in the range 20 – 120 μm (Figure 8). In another example, the nerve-regenerative

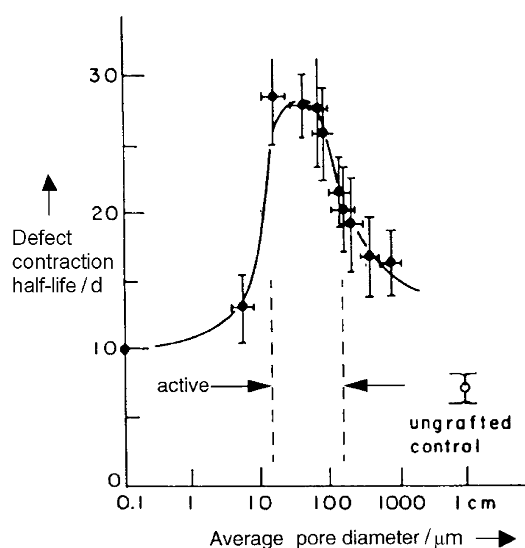

Figure 8. The contraction-blocking activity of a homologous series of scaffolds based on collagen – GAG graft copolymers is maximized inside a narrow range of the average pore diameter. Peak blocking activity has been shown when the pore diameter lies between 20 and 120 μm. (All other structural features, including pore volume fraction, were held constant for members of the series.) The dermis regeneration template (DRT) is located inside this critical range of the pore diameter. In this assay, the degree of contraction-blocking increases (contraction is delayed more) with an increase in the contraction half-life of the skin defect (wound) in the guinea pig model. (Reproduced from ref. [21], with permission).

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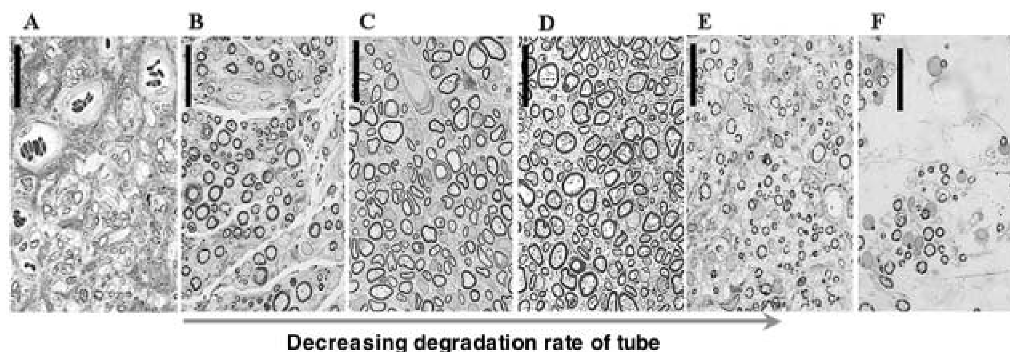

Figure 9. The quality of peripheral nerve regeneration is maximized when the implanted nerve chamber (tube) degrades in vivo at a critical rate. Cross-sections of five nerves that were regenerated by using porous type I collagen nerve chambers (tubes) with a degradation rate that decreased continuously from tube A to tube F. Morphometric data from rat sciatic nerves[54] have shown that nerves C and D had a significantly higher quality of regeneration than other members of the series of tubes. Bars: 25 μm. Images courtesy of M.I.T.

activity of collagen tubes (nerve chambers) peaked when the degradation rate of the implanted tube reached an intermediate value corresponding to a half-life for degradation of about 2–3 weeks (Figure 9). These results collectively indicate the important role that active scaffolds play in regeneration and the strong dependence of such activity on relatively small changes in scaffold structure.

The synthesis of scaffolds with regenerative activity by using basic physicochemical processes suggests that chemical manipulations that have been described in detail can continue to revolutionize the field of regenerative medicine.

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Keywords: biologically active scaffolds · biosynthesis · organ synthesis · regeneration templates · tissue synthesis

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