10

Cell Delivery and Recirculation

The Spheres themselves by motion do endure,
And they move on by Circulation too.

Katherine Philips, *To my Lucasia, in defense of declared Friendship*

Perhaps the simplest realization of tissue engineering involves the direct administration of a suspension of engineered cells—cells that have been isolated, characterized, manipulated, and amplified outside of the body. One can imagine engineering diverse and useful properties into the injected cells: functional enzymes, secretion of drugs, resistance to immune recognition, and growth control. We are most familiar with methods for manipulating the cell internal chemistry by introduction or removal of genes; for example, the first gene therapy experiments involved cells that were engineered to produce a deficient enzyme, adenine deaminase (see Chapter 2). But genes also encode systems that enable cell movement, cell mechanics, and cell adhesion. Conceivably, these systems can be modified to direct the interactions of an administered cell with its new host. For example, cell adhesion signals could be introduced to provide tissue targeting, cytoskeleton-associated proteins could be added to alter viscosity and deformability (in order to prolong circulation time), and motor proteins could be added to facilitate cell migration.

Ideally, cell fate would also be engineered, so that the cell would move to the appropriate location in the body, no matter how it was administered; for example, transfused liver cells would circulate in the blood and, eventually, crawl into the liver parenchyma. Cells find their place in developing organisms by a variety of chemotactic and adhesive signals, but can these same signaling mechanisms be engaged to target cells administered to an adult organism? We have already considered the critical role of cell movement in development in Chapter 3. In this chapter, the utility of cell trafficking in tissue engineering is approached by first considering the normal role of cell recirculation and trafficking within the adult organism.

10.1 Cell Movement within the Circulatory System

Most cells can be easily introduced into the body by intravenous injection or infusion. This procedure is particularly appropriate for cells that function within the circulation; for example, red blood cells (RBCs) and lymphocytes. The first blood transfusions into humans were performed by Jean-Baptiste Denis, a French physician, in 1667. This early appearance of transfusion is startling, since the circulatory system was described by William Harvey only a few decades earlier, in 1628. Denis used the blood of calves and lambs, choosing the animal that best matched the level of gentleness desired in the recipient. Early experience with transfusion was not entirely successful; Denis transfused blood into several patients, some on multiple occasions, and most experienced side effects such as shock and fever. Denis's procedure was blamed for the death of at least one patient, who was under treatment for madness, but further investigation revealed that the patient died of poisoning by arsenic, which was apparently administered by his abused wife (see an excellent history of blood and politics [1]). Transfusion was not widely used until the early 1900s, after Karl Landsteiner's discovery of blood types.

10.1.1 Circulation of Transfused Blood Cells

The utility of whole blood transfusion is now widely recognized. In addition, the fate of individual cells after introduction from a donor to a host is now well studied. Transfused RBCs circulate for long periods in normal human subjects. When RBCs are transfused into a patient, the cells disappear from the circulation linearly over 110 to 120 days following transfusion (Figure 10.1). When ^{51}Cr-labeled donor cells of type O were transfused into healthy recipients of type A, the persistence of the donor cells in the circulation could be followed by measuring radioactivity levels and products of hemolyzed cells of type O [2]. There were small but significant differences in the life span of the donor cells (life span = 114 ± 8 days for donor 1 cells in three patients; life span = 129 ± 5 days for donor 2 cells in the same three patients), which appeared to be related to the donor. These observations are consistent with the following hypothesis: the population of transfused RBCs has a uniform distribution of ages and each RBC has a finite life span of ~ 120 days.

How are RBCs removed from the circulation? Experimentally, it is difficult to tell a young RBC from an old RBC in a blood sample, but cells do differ in some properties such as membrane rigidity, suggesting that mechanical properties of cells may be an important signal for removal. While the survival curves in Figure 10.1 appear linear, the disappearance appears more curvilinear in other cases, suggesting that either the distribution of ages in the transfused cells is not uniform or cells are subject to a random elimination process.

Other blood-borne cells have a similar fate after transfusion, although the circulation lifetime of these cells varies greatly (Table 10.1). Some cells, such as the cells involved in immunological memory, may persist in the circulation for many decades.

Transfused cells (10^6/mm^3)

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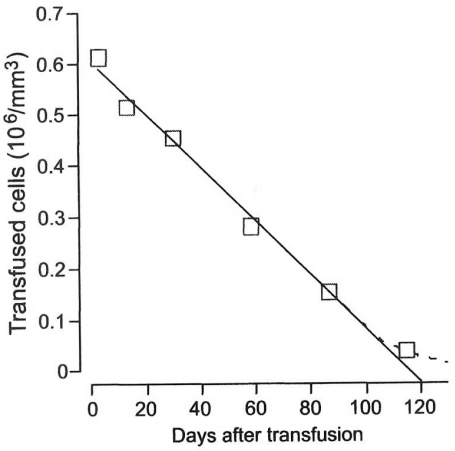


Figure 10.1. Survival of transfused red cells in a male adult. Until elimination of the cells is almost complete, the points fall on a slope which may be linear or slightly curvilinear. If the slope is assumed to be linear, mean cell life, estimated by extrapolation of the line to the time axis, is 114 days. The persistence of a few transfused cells beyond 114 days is due to variation in red cell life-span (redrawn from [4], p. 24).

The lung is an important site for trapping and loss of infused cells. Human lungs contain a tremendous number of capillaries, which carry deoxygenated blood from artery to vein in its passage over the alveolar wall. The average capillary diameter ($\sim 7.5 \pm 2.3\,\mu m$) is about the same size as a neutrophil ($6.8 \pm 0.8\,\mu m$) and a red blood cell ($7.5\,\mu m$). But neutrophils and RBCs differ dramatically in their mechanical properties, as described in Chapter 5; RBCs are more easily deformable. RBCs are therefore able to squeeze through the small lung capillaries more easily than neutrophils. RBCs move across the pulmonary capillary bed in ~ 1 second; transit of neutrophils through the lung capillaries is 60–100 times slower than that of RBCs [3]. Inflammatory

Table 10.1
Persistence of Transfused Cells in the Circulation

	Lifespan (days)	Notes
Red cells	120	
Platelets	8	
Neutrophils	0.3	45–55% are recoverable
Lymphocytes	~ 30	5–8% persist for > 270 days
TIL	Variable	98% disappear within a few hours

Collected from a variety of sources. TIL = tumor infiltrating lymphocyte.

mediators, and other factors that increase cell stiffness, further prolong neutrophil transit time.

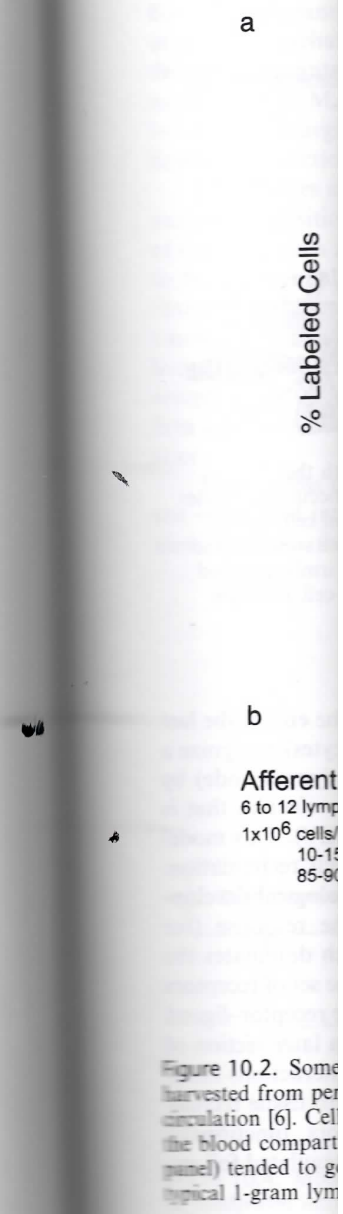
Only about 45–55% of the infused granulocytes are recoverable, even after intravenous administration, presumably because the other fraction is marginated in venules throughout the body [4]. Granulocytes that are labelled with ^{3}H-thymidine disappear from the bloodstream with a half-life of 7.6 hours, suggesting a daily production rate of 0.85×10^9/kg. It has been estimated that a 70 kg man has $\sim 4.2 \times 10^{11}$ marrow granulocytes of which 2×10^{10} are circulating ($\sim$ 5% of the total pool) [5]. Small lymphocytes have a life span of $\sim$ 1 month, but 5–8% of the cells have a lifespan of more than 9 months. In humans, some lymphocytes can live for years.

10.1.2 Cell Margination and Interaction with Endothelial Cells

Intravenously injected cells can sometimes find their way into extravascular body compartments. A simple example of blood-cell homing between blood and lymph compartments is shown in Figure 10.2 [6]. When lymphocytes were collected from blood and then injected intravenously, most of the injected cells remained in the blood (Figure 10.2a). In contrast, lymphocytes that were collected from the lymph tissue and then injected intravenously migrated back into the lymph (Figure 10.2a). Although the cells were circulating within the blood vessels immediately after injection in both cases, they were able to migrate out of the circulation and accumulate in other tissue locations; many of these cells can find their way back to their native tissue space (that is, the site where they were found). There are other well-known examples of this control of the fate of circulating cells; for example, infused hematopoeitic stem cells find their way back to the bone marrow.

How are intravenously infused cells able to escape from the blood? As described in Chapter 9, some cells have the remarkable ability to migrate out of the circulatory system into a specific tissue space, such as the skin or brain or heart. Since the blood vessel wall is the physical barrier between blood and tissue, it is logical to assume that control over cell exit from the circulation resides at the vessel wall. But how does interaction with specialized endothelium beds resident in varying tissues determine whether or not a cell will exit the circulation?

Circulating cells can associate with the blood vessel wall as a first step in the process of migrating out of the vessel and into local tissue. The sequential processes of margination, adhesion, and transmigration—which occurs frequently in the post-capillary venules—were described in Section 9.4.1 and are illustrated in Figure 10.3. In lymph nodes and specialized regions of the gut, lymphocytes in the circulation interact with an endothelium specialized for trafficking of lymphocytes; this specialized endothelium is found in post-capillary venules that are called high endothelial venules (HEV). Specialized endothelial cells of the HEV are recognized by lymphocytes through the selective binding interaction of cell adhesion receptors on both cell surfaces. The importance of cell–cell recognition is a common feature in regulation of cell



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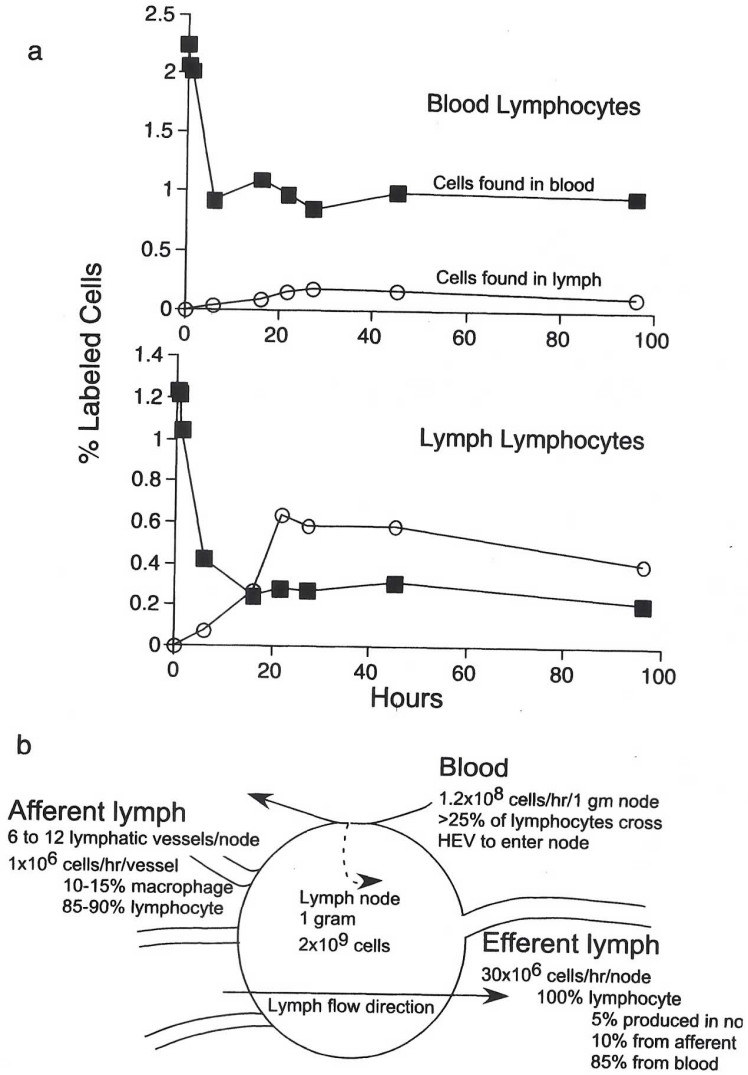

Figure 10.2. Some cells tend to return to their site of origin. (a) Cells were harvested from peripheral blood or lymph and then reinjected back into the circulation [6]. Cells harvested from blood (top panel) were found predominantly in the blood compartment after administration. Cells harvested from lymph (middle panel) tended to go back into the lymphatic system. (b) Traffic of lymphocytes in a typical 1-gram lymph node, redrawn from [43].

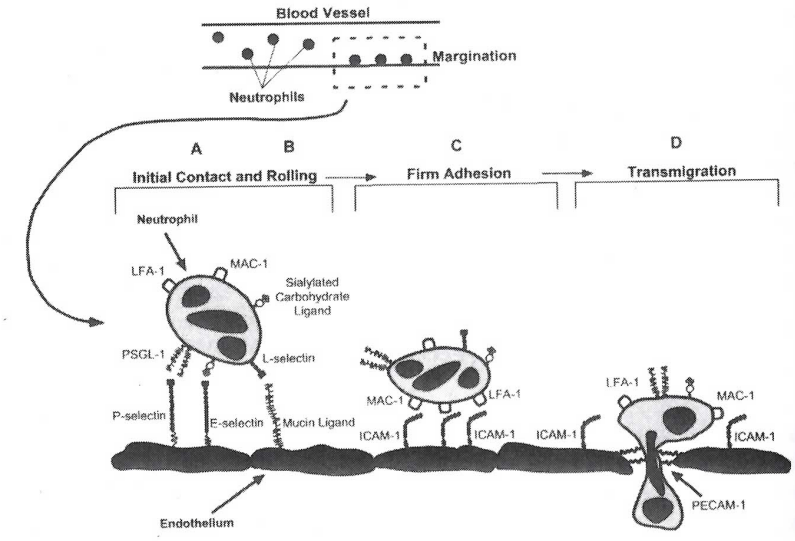

Figure 10.3. Cells within the circulation can become associated with the endothelium of the blood vessel. This process, called margination, occurs in a series of steps involving initial contact between the cell and the endothelial cell, loose adhesion (which is often characterized by the cell "rolling" along the surface), and firm adhesion which is resistant to the forces of the flowing fluid. Firmly attached cells can transmigrate through the vessel wall (see Chapter 9). Cell–cell adhesion molecules are involved with all of these events.

circulation. One part of the answer to the question posed at the end of the last paragraph seems to be this: circulating cells (such as lymphocytes) recognize a local tissue site for transmigration (such as the HEV of the lymph node) by molecular interactions with the specific set of cell adhesion molecules that is expressed on the surface of the local endothelium (such as HEV). This model can explain many of the features essential for control of cell recirculation: tissue sites can be made distinct according to their stage of biological development, their state of inflammation, or the type of immune response (for example, innate or acquired, allergic or inflammatory) which dominates the tissue microenvironment. Specificity is achieved by virtue of the set of receptors that are expressed on the local endothelium, leading to cognate receptor–ligand interactions. This point will be discussed more completely in a later section of this chapter. Control of this process is ongoing since these interactions influence expression of the cell surface receptors that intitially led to tissue recruitment in the first place (for example, chemokine receptors functionally expressed on the leukocyte cell membrane are typically down-regulated following ligand engagement by internalization).

Margination occurs continuously within the vascular system, so that the cells within the vasculature can be subdivided into two populations: the circu-

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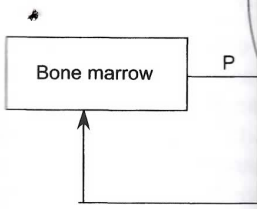

Figure 10.4. Model for cell
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described in [7].

lating fraction (f_C) and the marginated fraction (f_M) (see [2] for more detail). Individual cells can move between the two populations; that is, cells can become associated with the vessel wall and then return to the circulatory flow. The dynamics of movement of cells from one sub-population to the other can be characterized by rate constants, as illustrated in Figure 10.4; the rate constants describe the movement from the circulating to the marginated fraction, M, or return from the marginated to the circulating fraction, R. In addition, marginated and circulating cells can leave the vasculature to enter tissues; the fraction of cells in the tissue is f_T.

Fluid forces cause some cells to move to the periphery of the vessel cross-section, in position to interact with the endothelium. Certain characteristics of red cells, such as their tendency to aggregate or form rouleaux, cause them to be preferentially concentrated in the center of the vessel, with the result that other cells, such as neutrophils, are forced to the marginal position. Once a cell is at the vessel wall, the process of cell adhesion to the endothelium can be subdivided into phases (Figure 10.3). Primary adhesion, due to receptors on the tips of microvilli on the blood cell, is the initial interaction; firm adhesion, stabilized due to the engagement of integrin receptors, occurs later.

The process of cell adhesion to the vessel wall and transmigration out of the vessel and into the extravascular space is mediated by molecular interactions between the circulating cell and the endothelium. These interactions

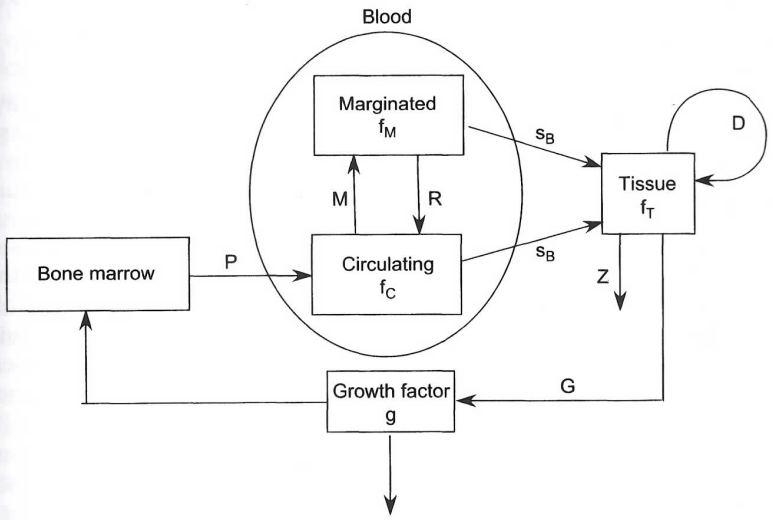

Figure 10.4. Model for cell margination and return. Cells within the circulation can be subdivided into two pools: circulating, C, and marginated, M. This schematic diagram illustrates a model for the fate of monocytes and neutrophils. The model is described in [7].

involve cell adhesion receptors on both the blood-borne cell and the endothelial cell (recall the discussion on adhesion receptors in Chapter 6). Our knowledge of the molecular identity of these receptors has increased dramatically in recent years, in part due to the explosion of genetic information available. For example, chemokines are a large family (over 50 members and growing) of small proteins (typically under 100 amino acids) with a degree of sequence homology that has permitted many of them to be discovered by cloning "in silico" (that is, by computer-based bioinformatics rather than the traditional laboratory approach). Although our list of the key players continues to grow with each discovery, the biological significance of these molecular ligands and their interactive roles under normal and pathological circumstances require definition.

One very interesting point regarding the circulating immune system is that normally there is a constitutive recirculation of lymphocytes between blood and tissue, referred to as immunosurveillance. For example, naïve T cells on the prowl for non-self or altered self antigens percolate through lymph nodes in hope of a rendezvous with their cognate antigen. Circulation of T cells through lymph nodes occurs continuously; large numbers of lymphocytes—entering from blood and from afferent lymphatics—move through lymph nodes every hour (Figure 10.2b). When antigen recognition does occur, it leads to T cell activation, which in turn alters the normal pattern of margination such that the activated T cell is now equipped to be efficiently recruited to the site of antigen origin in the periphery (for example, the skin where there is a site of injury and inflammation). Predictable changes in patterns of margination are known to occur in response to infection, organ transplantation, or allergic response. The modulation away from normal patterns of margination, measurable in terms of the fractional distribution of a distinct cellular subpopulation among blood and tissue, closely reflects the character of the perturbations occurring in a biological system.

10.1.3 Models for Cell Traffic Throughout the Body

Pharmacokinetic models have long been used to describe the dynamics of movement of molecules (particularly drugs or toxins) between tissues within the human body. Similar mathematical methods can be used to describe the fate of cell populations [7, 8]. Consider the case of neutrophils or monocytes, which circulate in the blood but can also marginate to the vessel wall and subsequently transmigrate into a tissue space. A simple mathematical model of blood cell trafficking throughout the body can be constructed [7]; in this model, cells can be in either the circulating, marginated, or tissue-associated state (Figure 10.4). The cells are produced at sites in extravascular tissues (that is, the bone marrow for neutrophils and monocytes) at a rate P; they may proliferate in tissues with a rate constant D; and they are destroyed with a rate constant Z. A model for the marginated, circulating, and tissue-associated cell subpopulations can be formulated from this description:

$$\frac{df_C}{dt} = P + P_g(g)$$

$$\frac{df_M}{dt} = Mf_C - (R$$

$$\frac{df_T}{dt} = S_B f_C + S_E$$

$$\frac{dg}{dt} = Gf_T - Kg$$

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10.2 Examples of Cell T

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$$\left.\begin{aligned}
\frac{df_C}{dt} &= P + P_g(g) + Rf_M - (M + S_B)f_C \qquad \text{(a)}\\
\frac{df_M}{dt} &= Mf_C - (R + S_B)f_M \qquad \text{(b)}\\
\frac{df_T}{dt} &= S_B f_C + S_B f_M + (D - Z)f_T \qquad \text{(c)}\\
\frac{dg}{dt} &= Gf_T - Kg \qquad \text{(d)}
\end{aligned}\right\} \qquad (10\text{-}1)$$

where g is the concentration of a self-stimulating growth factor that encourages cell production; this factor is produced by tissue cells (with a specific production rate G) and is eliminated from the tissue with a rate constant K (equal to ln(2)/tissue-half-life). Examples of the use of this set of equations for analysis of the extent of leukocyte margination have been presented [7], and are considered further in Exercise 10.1 at the end of the chapter. Importantly, these equations integrate patterns of leukocyte margination with other biologically related processes such as cellular destruction (apoptosis), which is ongoing and serves as a mechanism of cellular turnover that contributes to immune system responsiveness.

Similar pharmacokinetic models have been used to predict the effectiveness of adoptive immunotherapy [8].

10.2 Examples of Cell Trafficking between Body Compartments

10.2.1 Lymphocyte Recirculation

Lymphocytes are an essential element of the immune system, particularly with regard to their role in surveillance and recognition of foreign antigens (see [9]) . Lymphocytes are fantastically efficient at this function because they are continuously recirculating through tissue sites throughout the body—including the peripheral lymphoid organs and sites of likely infection, where foreign antigens are concentrated (Figures 10.2b and 10.5).

The patterns of recirculation are best known for T cells (Figure 10.5b). Naïve T cells (that is, T cells that have not yet responded to a specific antigen) arise from precursors in bone marrow and mature in the thymus before they enter the circulation; circulating human naïve cells can be identified by the presence of a high molecular weight isoform of CD45 (CD45A) on their surface. These circulating cells adhere to endothelial cells of the HEV within lymph nodes by a process involving L-selectin on the T cell and a molecule called peripheral node addressin (PNAd), initiating the first step of cell migration from the circulation into the tissue of the node, known as tethering. PNAd refers to any one of a group of endothelial sialomucins—(CD34, podocalixin, glyocsylation-dependent cell adhesion molecule 1 (GlyCAM-1), and sialyated glycoprotein of 200 kDa (sgp200) [10]. All of these molecules contain a sulfated sialyl-Lewis^X-like motif.

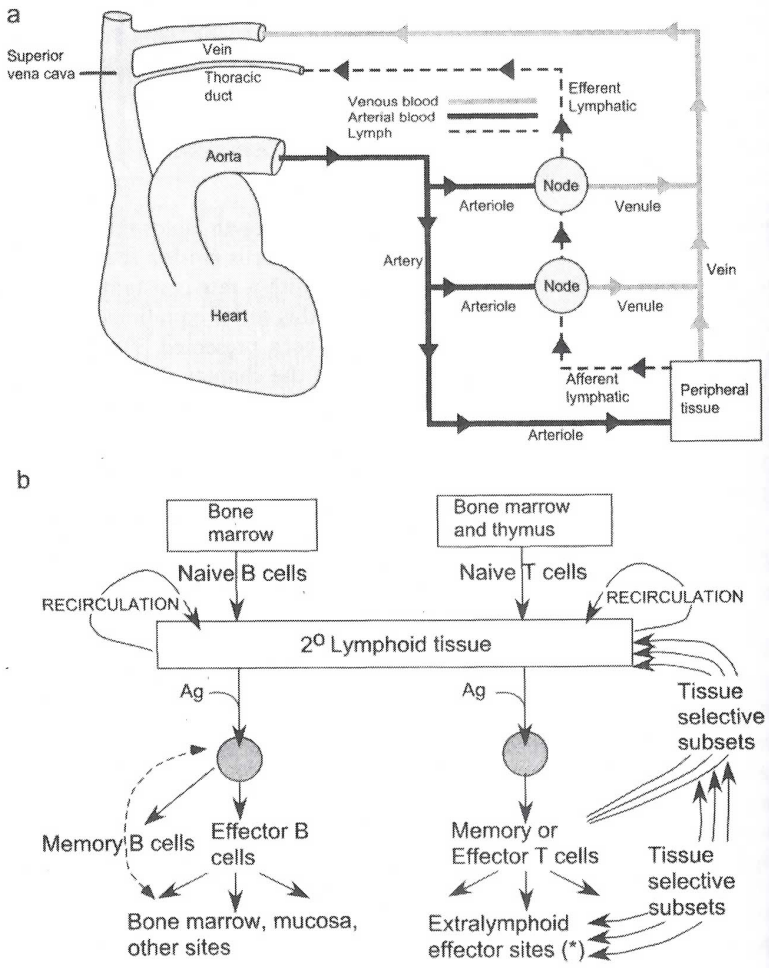

Figure 10.5. Schematic diagram of lymphocyte recirculation thoughout the body. (a) Lymphocytes follow a typical pattern in their circulation throughout the body. This pattern of circulation allows cells to observe, or sample, a large volume of the body, but also to be present for large periods of time within the lymphatic tissue, where they have more frequent encounters with foreign antigens associated with antigen-presenting cells. (b) Pathways for circulation and differentiation of B and T cells of the immune system. All cells move from their site of production, though the circulatory systems, and eventually into secondary and tertiary tissue sites. (*) includes skin, lung, intestine, etc.

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Table 10.2
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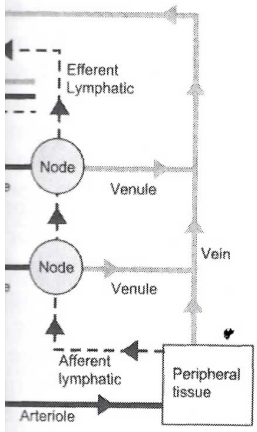
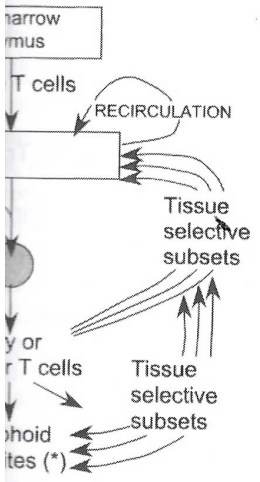
Activated T cell	CD11aCD CD49dCD CD49eCD CD49fCD CD44
Differentiated T cell	CD45RO

From [44].





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Recruitment into tissue includes additional steps that follow tethering: rolling, firm arrest, and transmigration across the endothelial vascular lining. During transmigration, the lymphocyte becomes deformed and flattened in order to physically squeeze between the membrane–membrane interface of juxtaposed endothelial cells; the endothelial cells also respond to the act of lymphocyte transmigration by alteration of cytoskeletal structure and distribution of surface molecules. The extravasated cell now travels through the lymph node, which is rich in foreign antigens that have been collected from peripheral tissues (Figure 10.5b). The antigen collection process in the lymph node is also dependent on many of the same migratory mechanisms. Antigen collection involves homing of antigen-laden dendritic cells from the antigen-rich periphery to the lymph node; this migration is guided by a distinct set of cognate binding interactions that are dependent on receptors found on the dendritic cell surface.

The physical localization of the lymphocyte within the lymph node is also dependent upon these interactions. If the T cell does not encounter its antigen within the node, it leaves via an efferent lymphatic vessel which carries the cell back to the venous circulation (Figures 10.2b and 10.5a). Amazingly, a recirculating T cell completes an excursion through the lymph node in approximately 1 hour. Immunoprotection depends upon the daily flux of naïve T cells through the compact, cellularly dense lymph tissue; the flux is high, providing many opportunities for antigen/T cell recognition.

A T cell that encounters an antigen during movement through the lymph tissue becomes activated. Activation causes the cell to enter the cell cycle and to increase the expression of certain surface molecules as well as their affinity for their counter-ligand (Table 10.2). These surface proteins increase cell adhesion to extracellular matrix (ECM) and, therefore, slow cell migration through the

Table 10.2
Surface Proteins Characteristic of T cells during Circulation and Differentiation

	Surface Proteins	Adhesion	Homing Receptors (T cell/Endothelium)
Naïve T cell	CD45A	Lymph node endothelium	L-selectin/ PNAd,GlyCAM-1 LFA-1/?
Activated T cell	CD11aCD18 (LFA-1) CD49dCD29 (VLA-4) CD49eCD29 (VLA-5) CD49fCD29 (VLA-6) CD44	Lymph node extracellular matrix	
Differentiated T cell	CD45RO	Inflammatory endothelium	VLA-4/VCAM-1 LFA-1/ICAM-1,2 CD44/Hylauronate
		Mucosal tissues	α4β7integrin/MadCAM-1 CD44/?

From [44].

lymph node, which is rich in ECM. Division and differentiation of activated T cells produce effector and memory cells; differentiated progeny retain high levels of expression of adhesion molecules, but these molecules do not exhibit high affinity. The differentiated cells are therefore released from the tissue and return to the circulation. Once in the circulation, however, the cells display a preferential adhesion to the endothelium of inflamed tissues and, therefore, an increased likelihood of migrating from the circulation at tissue sites where T cell effector activity is needed. Because the selective adhesiveness of T cells changes during activation and differentiation, cells at different stages of development have different fates in the body: naïve cells recirculate continuously through peripheral lymph tissue, activated cells commence proliferation and differentiation within the lymph node parenchyma, and memory/effector cells target inflamed tissue sites. This process of selective lymphocyte movement, which occurs in coordination with the activated molecular surface of the vascular endothelium in peripheral tissues, is called homing.

How do T cells recognize particular locations in a tissue? As described above, T cell migration from the circulation occurs preferentially within specialized vessels with protruding endothelial cell walls called HEVs. HEVs were first found in lymph nodes, but are also present in Peyer's patches of the gut. Endothelial cells of the HEV are particularly adhesive to T cells, so that cells passing through these vessels will cling briefly to the endothelium during transit. This brief halting of cell movement (descriptively referred to as "tethering") creates an opportunity for the T cell to roll along the vascular surface and concomitantly sample the repertory of heparin-bound chemokines present on that surface. This rolling/sampling process can trigger firm T cell adherence to the wall, which leads to extravasation.

Lymphocytes are distinguished by the molecular composition of their outer membranes; some of the distinct molecules of each lymphocyte are involved in the process of recognition of endothelium and homing. Molecules that are involved in this process have been described for a number of different lymphocyte subpopulations (Table 10.3).

10.2.2 Peripheral Blood Stem Cell Infusions

The delivery of extremely high doses of chemotherapy agents can improve the survival of patients with certain kinds of tumors. Unfortunately, high-dose chemotherapy is often associated with devastating toxicity, particularly the suppression of production of normal bone marrow tissue components. But suppressed bone marrow can be repopulated by transfusion of cryopreserved bone marrow stem cells. The infused marrow stem cells may be autologous (harvested from the patient prior to the high dose chemotherapy) or allogeneic (harvested from a donor). Alternatively, marrow stem cells can be obtained from neonatal umbilical cord blood or from peripheral blood following mobilization, a process in which chemotherapy drugs or cytokines are used to induce the release of stem cells from the marrow into the peripheral blood.

Table 10.3
Adhesion Decision Cascades for Lymphocyte Homing

Endothelial Cell	Counter-receptor on Lymphocytes

Table 10.3
Adhesion Decision Cascades for Lymphocyte Homing

Lymphocytes	Target Destination	Endothelial Cell Adhesion Molecule	Counter-receptor on Lymphocytes			
			Contact	Rolling	Arrest	Diapedesis
Naïve B or T cells	Peyer's patch	MAdCAM MAdCAM-1 ICAM	L-selectin---	--------- -- α4β7-----	---------- ----	LFA1 ----------
Naive B or T cells	Peripheral lymph node	PNAd ??? ICAMS	L-selectin---	--------- --???-------	---------- ----	LFA1 ----------
Skin-homing memory cells	Skin	E-selectin VCAM-1 ICAMs	CLA--------	-------- ----- α4β1--	----------	LFA1 ----------
Nonmucosal memory cells or blasts	Inflamed CNS, heart, others	??? VCAM-1 ICAMs	??? ---------	-------- ----- α4β1--	----------	LFA1 ----------

More detailed information is available in review article [10].

Because of the disadvantages of current techniques for bone marrow harvest, including the complicated and uncomfortable procedure of collecting 1 to 2 L of bone marrow from patients, there is substantial interest in the development of cell culture systems that would permit the expansion of bone marrow outside the body (see [5] for a review of this technology).

10.2.3 T cell Therapies

Great progress has been made over the past two decades in the development of therapies that employ adoptive transfer of activated or engineered T cells [11]. Adoptive transfer of cells has many advantages over vaccine approaches in which a patient's own immune system is stimulated to produce active T cells. In cell transfer therapy, large numbers of cells can be administered; the cells can be activated *ex vivo*, allowing for control of the activation process; the exact cells that are required for therapy can be identified; and the host can be manipulated prior to transfer in order to optimize therapy.

The first evidence that the immune system could be manipulated to treat solid tumors in humans was achieved by high-dose delivery of the cytokine interleukin-2 (IL-2). IL-2 has a diverse spectrum of activities in the immune system, including stimulating the proliferation of activated lymphocytes. High-dose IL-2 led to tumor regression in a fraction of patients with metastatic melanoma (15% of patients experienced regression) and metastatic renal cell cancer (19%). IL-2 has no direct effect on tumor cell growth; the regression must therefore be due to an influence of the cytokine on cytotoxic immune cells. In this case, the IL-2 is acting on cells that already reside in the patient; responses, therefore, are limited only to patients who harbor enough cells that are capable of responding to the cytokine. This approach is also limited by the toxicity of high-dose IL-2 therapy to other cells and tissues in patients.

To further enhance the beneficial activity of IL-2, it can be administered together with an additional population of activated lymphocytes. In this approach, the activity of IL-2 on the patient's immune system is augmented by providing a large number of new cells that are capable of mediating tumour regression. Success of this cell transfer therapy depends on (1) the ability to identify cell populations that will be effective at mediating tumor regression in the patient, and (2) the ability to amplify the cell population *ex vivo*. Often, the precursor cells are collected from within the tumor itself; these cells are called tumor-infiltrating lymphocytes (TILs).

The circulation of lymphocytes after infusion depends on the source of the cells and can be influenced by treatment with cytokines. In an early study, TILs were labeled with ^{111}In (10^{10} cells) and infused together with IL-2 therapy, as tolerated [12]. In these patients TILs disappeared rapidly from the circulation: less than 2% were found after 1–2 hr. Initially, TILs were localized in the lung (30%), liver (30%), and spleen (10%). Liver and spleen levels were stable over seven days, while lung levels dropped to ~ 10% after one day. Tumor uptake increased over time, with maximum uptake occurring in the period of 48–72 h following infusion; the tumor localization index (ratio of cells in tumor/normal

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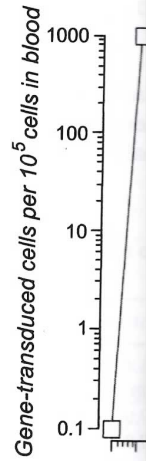

Figure 10.6. TIL cell elimin
into a patient. A semi-quant
function of time following T

tissue) varied between 1 and 40 over the first 10 days after infusion. Normal, unactivated lymphocytes appear predominantly in the liver, lung, and spleen after infusion, with lung activity clearing within 24 h and liver and spleen activity stable for 96 h. The main differences between normal lymphocytes and TILs are (1) that some TILs remain in the liver for an extended period, and (2) that normal lymphocytes can traffic, but do not accumulate in the skin (whereas the TILs localize to the melanoma sites).

In a report of tumor-infiltrating cell infusions in patients, five patients were treated with TILs that had been transduced with a retroviral gene as a marker [13]. TILs, harvested from resected tumors and propogated in culture, were transduced with the retroviral vector in culture. Up to 2×10^{11} cells were infused into the patient over a 30–60 min period. Infusions of cells were followed by infusion of IL-2 . Cells were found in the circulation of patients for three weeks in all patients (up to two months in two patients). Cells were found at tumor sites up to 60 days following infusion. An example of the number of cells present in the peripheral blood of one of the patients is shown in Figure 10.6.

Other T cell therapies are already available for clinical use. For example, extracorporeal photochemotherapy or photophoresis is used to treat patients with cutaneous T cell lymphoma [14]. In photophoresis, ultraviolet light is focused on the blood of lymphoma patients as it flows through a serpentine-shaped plastic chamber outside of the body. Exposure to the light causes the tumorigenic blood-borne T lymphocytes to enter apoptosis; debris resulting

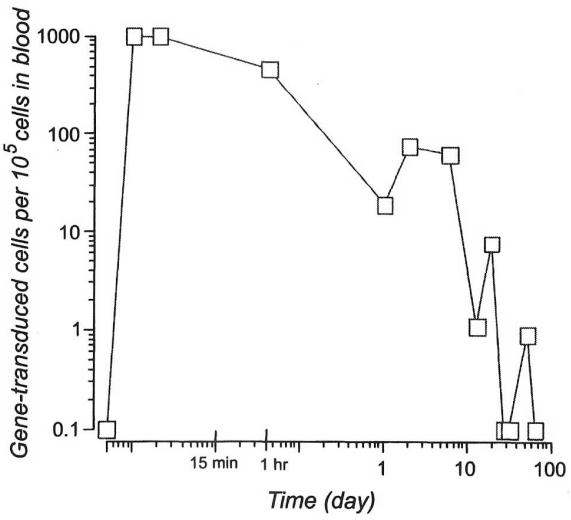

Figure 10.6. TIL cell elimination from the peripheral blood following transfusion into a patient. A semi-quantitative determination of cell number is shown as a function of time following TIL infusion (adapted from Figure 5 of [13]).

from cell death is taken up in part by circulating monocytes. Exposure of monocytes to the plastic surface of the extracorporeal chamber also seems to induce their differentiation to dendritic cells, which are effective in antigen presentation [45]. The monocytes are reintroduced to the blood of the patient; it is believed that immune activation accounts for full or partial recovery in a substantial percentage of cases. It is suspected, but not proven, that this therapy results in the activation of anti-tumor killer T cells that attack the tumorigenic T cell at the site of the skin lesion.

Animal models have also been used to study the fate of transfused cells. For example, mouse natural killer (NK) cells were activated with IL-2 and then injected into the tail vein of mice with the subcutaneous FSaII fibrosarcoma [15]. Positron emission tomography (PET) was used to measure the biodistribution of cells labelled with [^{11}C]methyl iodide for animals that received injections of 10^7 activated NK cells or control (unactivated) cells. Retention of cells within the tumor was increased, compared to control cells, during the first 30–60 min following injection (Table 10.4). It is not clear whether the increased retention at the tumor site is due to increased rigidity of the activated cells, which increases mechanical trapping of cells in the tumor vasculature, or to increased interactions of cells with the tumor endothelium, although the vessels in the tumor appear to be large enough to permit even rigid cells to pass through easily. It is now possible to learn about the molecular details of interaction in systems such as this one; for example, the expression of adhesion molecules and chemokine receptors can be measured on the entrapped cells, using molecular tools such as quantitative RT-PCR.

10.2.4 Tumor Metastasis

In addition to the movement of blood cells out of vessels, migration from the interior of the vessel to the outside has also been observed for other types of cells. One unfortunate example is tumor metastasis; malignant cells sometimes

Table 10.4
Distribution of Activated NK cells Injected into the Vasculature of a Tumor[a]

	Activated NK cells	Control cells
Tumor	15.3 ± 4.9	3.4 ± 0.2
Lungs	28.3 ± 5.1	21.6 ± 0.9
Liver + spleen	15.3 ± 1.6	17.0 ± 0.6
Other	41.9 ± 3.5	57.9 ± 1.6

[a]Percentage of total injected cells at various sites 30–60 min after injection of 10^7 cells into the tail vein of mice with subcutaneous tumor in the tail. Data from [15]. Retention of activated cells within the tumor is enhanced, but the mechanisms contributing to this enhancement are still unknown.

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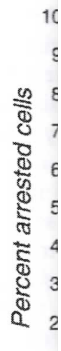

Figure 10.7. Dynamic
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enter the circulation and then re-emerge from vessels at a distant tissue site. Tumor cell egress from the vasculature has been visualized using the chorioallantonic membrane (CAM) [16, 17], which allows determination of the time course of tumor cell migration from the vasculature after injection (Figure 10.7). Within 2 min of injection, all of the tumor cells were arrested, probably due to size restrictions since the cells are significantly larger (~ 20 μm) than the plexus channels in the CAM (~ 9 μm). In these experiments, there was almost no evidence that tumor cells were arrested due to adhesion to the vessel wall. (The mechanisms responsible for cell arrest are still in debate [18].) Following arrest, the cells may move slowly with the flow within the local vasculature, while undergoing extensive deformation and changes in morphology. The cells could then extravasate from the vessel into the extravascular space; this occurred primarily within the period of 5–10 hr following injection (Figure 10.7). The rate of cell migration into the extravascular space was approximately 1 μm/hr (which is consistent with rates of tumor cell migration, Table 7.2). No cell destruction was observed during the periods of observation, but this may be due to the milder flow conditions in the chick membrane (no white cells in the blood, low-velocity blood flow). The majority of potentially metastatic cells (up to 99%) are destroyed in the mouse lung following injection. In the CAM, cell division did not occur prior to extravasation, but did occur after; the cells tended to divide to form micrometastases around the vessels.

Since cell adhesion receptors mediate the interaction of blood-borne cells with the vessel wall, and since this interaction is essential for transmigration

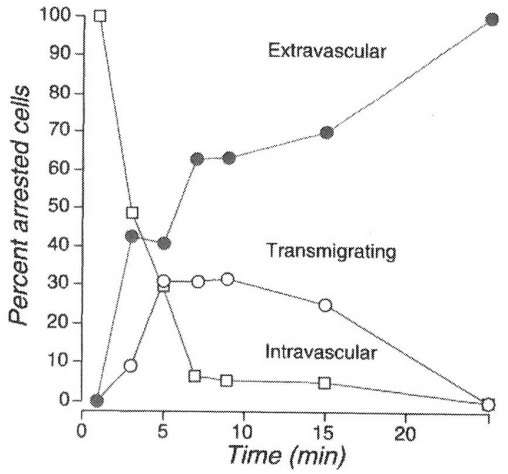

Figure 10.7. Dynamics of transmigration in a model system. The dynamics of tumor cell migration out of the circulatory system can be studied in model systems such as the chick chorioallantoic membrane (CAM). In this experiment, the tumor cells are initially within the blood vessels, but they quickly adhere to vessel walls and transmigrate into the extravascular space.

into tissues, it might be possible to block tissue localization by using agents that block cell adhesion. One early report testing this hypothesis provides evidence for reduction in colonization of the lung of mice with the coadministration of GRGDS, a peptide that should inhibit tumor cell binding to integrin receptors, and B16-F10 murine melanoma cells [19]. Reduction in colonization requires high doses (~ 3 mg) of GRGDS per mouse, which leads to a blood concentration of 3 mM. The low efficiency of this approach may be due to the relative low affinity of blocking peptides with cell adhesion receptors. For example, the affinity of fibronectin for its receptor is $K_d = 0.8 \times 10^{-6}$ M (recall discussion in Chapter 6). Still, at least in some experimental models, it is possible to reduce colonization with high doses of peptide. It is likely that more can be done in this regard in the future by enagaging multiple arms of the immune system; for example, down-regulation of inflammatory mediators (for example, TNF) might be useful as an adjuvant therapy to this blocking peptide strategy.

10.3 Microchimerism

A common method in tissue engineering is the transplantation of cells from a donor into a host. In this chapter so far, we have been considering the introduction of cells by infusion into the circulatory system. It is clear from previous experience that infused cells can enter tissues, and that the dispersion and retention of cells will depend on properties of the transplanted cell population as well as properties of the host. How does this information guide our thinking about the long-term consequences of tissue engineering in human patients?

It is now known that transplanted cells, even when introduced to the host by means other than infusion, can migrate to tissues of the recipient that are distant from their site of introduction and that they can persist at these sites for long periods of time. This new appreciation of the frequency and magnitude of mixing of cells between the donor tissue and the host—a slow process of intermingling that results in a "mixed" state that is called microchimerism—has emerged from observations in two different clinical settings: cells from transplanted solid organs that mix into other tissues of a transplant recipient [20], and cells from a fetus that persist within tissues of the mother [21].

The modern age of organ transplantation began in the middle of the twentieth century (see Chapter 1). It has been known for several decades now that the transplanted organ becomes chimeric: cells from the host migrate into the transplanted organ and function in that environment. For example, reticuloendothelial cells of the host migrate into transplanted livers and replace donor cells, creating a transplanted organ that has abundant cells from the donor (liver parenchymal cells, endothelial cells) and cells from the host (reticuloendothelial cells). It is also well known that the migration of donor immune cells from the transplant can initiate pathological changes in the host through a spectrum of diseases known as graft-versus-host disease (GVHD). But, in 1992, Starzl and his colleagues demonstrated that chimerism

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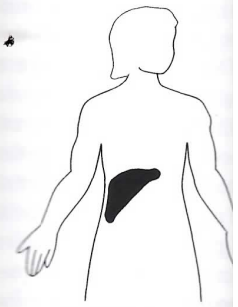

Transplantation of liver
(black) into host (white)

Figure 10.8. Developmen
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migrate into the donor or
into the host (right panel).

after solid organ transplantation is, in fact, systemic [20]. In long-term recipients of liver and kidney transplants, donor cells could be found in the host skin, blood, lymph nodes, heart, and other organs (Figure 10.8).

In microchimeric patients, after solid organ transplantation, many of the systemic donor cells have been identified as antigen-presenting cells. Since this state of microchimerism was found in long-term transplant survivors, it was hypothesized that the microchimeric state was an important step in the dynamics of immune response to a transplanted organ. The patient now has cells from two immune systems (host and donor) and these cells can both be active; if the response of the donor cells dominates, then GVHD can develop, whereas if the response of the host immune system dominates, then organ rejection can occur. Perhaps the microchimeric state represents a piece of an equilibrium condition that is established between immune response of the host and donor, in which a balance has been obtained that produces neither GVHD nor rejection. This balance, which leads to organ acceptance or immunological tolerance, may be facilitated by the cellular events observed in microchimerism. In this hypothesis, immunosuppression therapy, which is given to transplant recipients, may serve as a screen that allows microchimerism to prosper, producing an ultimate state of immune balance in which organ acceptance requires no additional immunosuppressive drugs. One prediction of this hypothesis is that immune tolerance may be enhanced by the intentional introduction of donor leukocytes into the patient. Animal models of rejection and microchimerism after skin grafts suggest that maturity of the host immune system and function of the donor cells are both important variables in the prediction of rejection or tolerance outcomes [22].

Although the overall hypothesis—that microchimerism enhances the process of tolerance—is still controversial [23], it is clear that substantial exchange

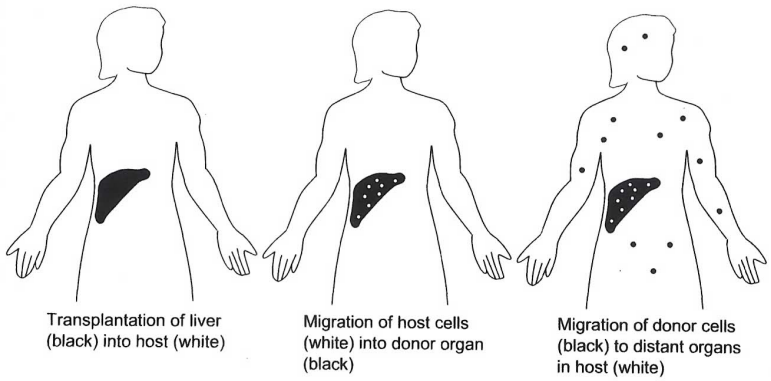

Figure 10.8. Development of microchimerism. After transplantation of a solid organ (here, a "black" liver into a "white" host, left panel), cells from the host can migrate into the donor organ (middle panel) and cells from the organ can migrate into the host (right panel).

of cells can occur between transplanted solid organs and the host. This observation may have important consequences for clinical use of tissue-engineered products. Indeed, in order for graft acceptance to succeed, transplanted organs must become perfused by the recipient's blood, itself an invitation to the exchange of cells.

Cells of fetal origin can persist for long periods (perhaps a lifetime) in the tissues of a mother. Since women experience an increased incidence of a variety of autoimmune diseases (such as systemic sclerosis, primary biliary cirrhosis, and Sjögrens syndrome), it has been hypothesized that the microchimeric state produced by pregnancy may increase the risk for autoimmune diseases. Again, studies on both sides of the hypothesis have been reported and the relation between microchimerism and autoimmune disease remains controversial [22].

10.4 Cell Penetration into Three-Dimensional Tissues

The physics of cell movement through the circulation is well known; the physical forces and chemical components of transmigration across an endothelial barrier are emerging (as outlined in the sections above). But how do cells migrate through a three-dimensional tissue space which is filled with other cells and, potentially, a dense extracellular matrix in the interstitial space?

Some motile cells can migrate through reconstituted extracellular matrix gels, which may reflect the ability of cells to migrate through solid tissues *in vivo*. It is well known that such movement is required physiologically, since, once extravasation occurs, monocytes and T cells must make their way through the ECM guided in part by chemokines produced by the underlying epithelia (for example, keratinocytes in skin) in order to eventually find their way to the afferent lymphatic which leads to the local draining lymph node. Mechanistically, human neutrophils move rapidly within three-dimensional gels of rat tail collagen; the rate of movement depends on the nature of the surrounding gel, in particular on the density of the ECM gel [24] (see Figure 7.6a). This observation may have important implications. In fulfilling their role in the immune response, neutrophils migrate out of capillaries, through tissue, to sites of infection. Perhaps, within the tissue, cell speed is influenced by the level of ECM hydration. The local tissue edema that accompanies inflammation may be a significant factor, modulating the rate of neutrophil infiltration. Similar factors may influence the speed of leukocyte migration—and therefore the efficiency of immune cell surveillance—in mucus secretions. For these reasons, experimental techniques for quantifying cell movement in three-dimensional gels and tissues have been developed as a first step in evaluating the mechanism by which cells migrate through tissues [24, 25]. Much about the process of cell migration through complex media remains unknown; for example, the relationship between the stiffness of the migrating cell—and changes in stiffness due to dynamic changes in the cytoskeletal architecture at the intracellular level—and the rate of cell migration is still unknown.

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Motile cells within three-dimensional gels exhibit a random migration process that is similar to cell migration on a surface. In some systems the similarity between the trajectories of motile cells and particles undergoing Brownian motion is striking, suggesting that diffusion might be responsible for the movement that is quantified by direct observation in three-dimensional gels. The diffusion coefficient for a spherical particle in water can be estimated from the Stokes–Einstein equation

$$D_w = \frac{kT}{6\pi\eta r} \tag{10-2}$$

where D_w is the diffusion coefficient of the particle in water, k is Boltzman's constant, T is the absolute temperature, η is the viscosity of water at T, and r is the radius of the particle. For a particle with a radius of 5 μm diffusing in water at 37°C, Equation 10-2 gives D_w of 6×10^{-10} cm^2/s, not much lower than the smallest random motility coefficient measured in common experimental systems (that is, 16×10^{-10} cm^2/s) (Figure 7.6a). But red blood cells, which are similar in size to neutrophils, never move after suspension in a collagen gel, even when the experimental duration is long. Furthermore, if diffusion were responsible for cell movement, the diffusion coefficient would monotonically decrease with increasing collagen concentration due to the increase in viscosity and/or viscoelasticity; however, motility coefficients show a maximum in the random motility coefficient at an intermediate collagen concentration (Figure 7.6a). On the basis of these observations it is reasonable to speculate that the fibers in the gel prevent diffusion of cell-sized particles; there must be some active interaction between cells and ECM fibers that enables these cells to traverse the gel.

Random motility coefficients for human neutrophils within gels of rat tail collagen varied from 1.6 to 13×10^{-9} cm^2/s (Figure 7.6a). How does this compare to the rate of cell migration on a solid surface? Two alternative quantitative assays have previously been employed to estimate this coefficient for neutrophils. Using an under-agarose assay, in which cells are attached to a two-dimensional substrate, μ was $\sim 10^{-8}$ cm^2/s [26], near the maximum observed in this study; using a filter assay, where cells migrate through a three-dimensional mesh of synthetic fibers, μ was $\sim 10^{-9}$ cm^2/s [27], near the minimum observed in collagen gels. While there are potentially important quantitative differences between observed migration rates in these assay systems, it is interesting that these three very different techniques yield similar values for the random motility coefficient.

Several previous reports examining the relationship between adhesion and cell motility on surfaces suggest that the strength of a cell–surface adhesion must be at an optimal level for cell migration (recall discussion in Chapter 7). If the adhesive strength is well above this optimum, cells will become fixed to the surface and hence will not migrate. If cell–surface adhesion falls much below the optimal level, the cell will not be able to gain sufficient traction to translocate across the surface. There is probably also an optimal adhesiveness for neutrophils migrating in three-dimensional materials. In the collagen gel sys-

tems, below a concentration of 0.1 mg/mL, most neutrophils rapidly sink through the gel; at concentrations above 0.7 mg/mL, the majority of neutrophils are immobilized (although neutrophils were seen to extend and retract pseudopodia in these high concentration gels, they were unable to make significant displacements from their initial positions). Between these two extremes, neutrophils exhibit maximal motility at a collagen concentration of 0.3 mg/mL (Figure 7.7). Inter-collagen fiber spacing is approximately equal to a neutrophil diameter ($\sim 10\,\mu m$) at 0.1 mg/mL, and decreases as the collagen concentration is raised [28]. These previous results suggest that when the collagen concentration is increased above the optimal level (0.3 mg/mL), the strength of the cell–fiber interactions becomes stronger due to the increased number of collagen fibers surrounding the cell. Analogously, at lower collagen concentrations where there are fewer fibers, the cell–fiber adhesive strength is lower (and the speed of migration can be influenced by adding antibodies against cell adhesion receptors [29]). Both of these situations result in a decrease in the effectiveness with which cells are able to migrate through the gel. Cells in solid tissues experience a more complex environment—they are surrounded by a variety of ECM components as well as adjacent cell surfaces—but some aspects of this mechanism probably act in tissues as well.

How do these results compare to the rates of cell migration in tissues in a living organism? This question is difficult to answer, since there are few reliable, quantitative measurements of the rate of cell migration through a tissue space. In one model system, the rate of migration of pigmented retinal cells within a neural cell aggregate was determined [30]. The rate of cell movement in this situation was much slower than rates of cell movement on surfaces or within three-dimensional ECM gels (Table 10.5).

Cells that have been transplanted into solid tissue can potentially migrate, reducing their effectiveness at the local site and potentially leading to unwanted side effects of the cell delivery. It may also be possible to learn about rates of cell migration in tissues by studying the fates of cells transplanted into a living organ. Neuronal cell transplantation has been an important experimental tool for many years. As early as 1917, pieces of neural tissue were transplanted into defects in the neocortex of adult animals [31] or introduced through cannulae

Table 10.5
Random Motility Coefficient for Cells in Different Experimental Systems

	Motility Coefficient, μ or Diffusion Coefficient, D (cm^2/s)	Reference
Oxygen in water	1×10^{-5}	[41]
Protein in water	1×10^{-7}	[41]
Neutrophil in ECM gel	1×10^{-8}	[24]
Fibroblasts on glass surface	1×10^{-9}	[42]
Pigmented retinal cell in neural aggregate	5×10^{-12}	[30]

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[32]. More recently, techniques for the transplantation of dissociated neural tissues have been introduced and studied extensively [33–38]. An excellent review of the technical procedures involved in neural transplantation of tissue pieces, dissociated tissue fragments, and dissociated cell suspensions is available [39].

Cells within the subventricular zone of the lateral ventricle in adult mice continue to proliferate. Using genetically labeled cells from donor transgenic mice, which were transplanted into immunologically identical hosts, as well as endogenous cells labeled with microinjected ^{3}H-thymidine, these proliferating cells were found to migrate into the olfactory bulb [40]. The speed of migration was estimated as 30 μm/hr (see Table 7.2 for comparisons). When examined during the period 6– 30 days following transplantation, the cells had migrated up to 5 mm. Although it is difficult to make quantitative comparisons with the rates of migration in model systems, which are more easily quantified, it is clear that cells can migrate long distances after transplantation into a solid tissue.

Summary

- Cells can be introduced into a recipient by infusion; this technique has been used successfully for many decades, and the lifetime and distribution of some cell populations after infusion are now well known.
- Cells within the circulation can transmigrate through endothelial barriers to reach a local tissue site. The process of transmigration occurs in a series of steps; some of the molecular participants in adhesion and transmigration have been discovered.
- Cells in the circulation can find their way to specific tissue sites; cells rely in part on the molecular composition of the local endothelium to determine their location in the body.
- Cells that are transplanted into a host can persist for long periods of time under the right conditions. The long-term persistence of donor organ cells that are widely distributed in the host is evidence of the capacity of transplanted cells to migrate long distances and to survive.

Acknowledgement

This chapter benefited greatly from editing and suggestions from Dr. Martin S. Kluger.

11

Delivery of Molecular Agents in Tissue Engineering

All truths wait in all things;
They neither hasten their own delivery, nor resist it . . .

Walt Whitman, *Leaves of Grass*

The previous chapter provided some examples of tissue engineering, in which cells that were isolated and engineered outside of the body are introduced into a patient by direct injection of a cell suspension, typically into the circulatory system. But the field of tissue engineering also points to treatments that are conceptually different from variations on cell transfusion technology; tissue engineering promises the regrowth of adult tissue structure through application of engineered cells and synthetic materials [2]. In support of this broad claim, the field of tissue engineering can point to some initial successes. For example, synthetic materials are now available that accelerate healing of burns and skin ulcers [3]. In addition, *in vitro* cell culture methods now allow the amplification of a patient's own cells for cartilage repair or bone marrow transplantation.

But major obstacles to the widespread application of tissue engineering remain. Tissue engineers have not yet learned how to reproduce complex tissue architectures, such as vascular networks, which are essential for the normal function of many tissues. In fact, the tissue engineering concepts that have been demonstrated in the laboratory to date involve arrangements of cells and materials into precursor tissues (or neotissues) that develop according to natural processes that are already present within the cells or the materials at the time of implantation (Table 11.1). These methods may be suitable for production of some tissues in which either the structure is relatively homogeneous (such as cartilage, in which a tissue structure can reform after the implantation of chondrocytes into a tissue defect) or the structure develops naturally (such as in some tissue-engineered skin, in which the stratified epithelium develops naturally by culturing at an air–liquid interface [4]).

The engineering of many tissue structures—such as the branching architectures found in many tissues or the intricate network architecture of the nervous system—will probably require methods for introducing and changing molecular signals during the process of neo-tissue development. For example,

Table 11.1
Stages of Development ir

Tissue	Neotissue
Vascular networks	Human dern endothelial c degradable P *vivo*)
	Flk1+ cells embryonic st 3D collagen *in vivo*)
	Bcl-2 transdu umbilical vei within a 3D matrix (*in vi*
Bone	Bone morph PLA-DX-PE matrix (*in viv*
	Osteogenic ce presence of T
Cartilage	Free and lipo TGF-β1 in fi
Peripheral nerve	Fibrin matric peptides (*in v*
Central nervous tissue	PLGA micros within brain c *vitro* and *in vi*
Thymic organ	Thymic strom progenitors w coated carbon

Abbreviations: transforming (VEGF), basic fibroblast gr growth factor (NGF), epide

Table 11.1
Stages of Development in Selected Examples of Tissue Engineering

Tissue	Neotissue	Stages of Development	Agents of Interest	Ref.
Vascular networks	Human dermal microvascular endothelial cells within a degradable PLLA matrix (*in vivo*)	Dispersed cells (day 1) Tubular structures (day 5) Functional microvessels (day 7)		[84]
	Flk1+ cells derived from embryonic stem cells within a 3D collagen matrix (*in vitro* and *in vivo*)	Aggregates (day 0) Process formation (day 3) Organized tubular structures formed of multiple cell lineages (day 5)	VEGF	[85]
	Bcl-2 transduced human umbilical vein endothelial cells within a 3D collagen/fibronectin matrix (*in vitro* and *in vivo*)	Organization into cords (day 1) Formation of vascular structures and complex multicellular walls (31 to 60 days)		[86]
Bone	Bone morphogenetic protein in PLA-DX-PEG copolymer matrix (*in vivo*)	Repair of 2 mm bone defects (time and dose-dependent over 2–4 weeks)	rhBMP-2	[87]
	Osteogenic cells cultured in presence of TGF-β1 (*in vitro*)	Formation of 3D aggregates (1–2 days) Production of bone minerals (3–10 days)	TGF-β1	[88]
Cartilage	Free and liposome-encapsulated TGF-β1 in fibrin matrix (*in vivo*)	Migration of mesenchymal cells into matrix (days) Differentiation of cells into mature chondrocytes (weeks)	TGF-β1	[89]
Peripheral nerve	Fibrin matrices with bioactive peptides (*in vivo*)	Regrowth of axons into gap of severed dorsal root (4 weeks)		[90]
Central nervous tissue	PLGA microspheres dispersed within brain cell aggregates (*in vitro* and *in vivo*)	Release of NGF and accumulation in tissue (hours to days) Differentiation (days)	NGF	[11]
Thymic organ	Thymic stromal cells and progenitors within 3D tantalum-coated carbon matrix (*in vivo*)	Formation of confluent stromal layer on matrix (14 days) Co-culture with hematopoietic progenitors to produce T cells (14 days)		[91]

Abbreviations: transforming growth factor β (TGF-β), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), bone morphogenic protein (BMP), nerve growth factor (NGF), epidermal growth factor (EGF).

it is well known that chemical gradients of factors known as morphogens induce the formation of structures during development [5, 6]; some of the attributes of morphogens were introduced in Chapter 3. In addition, changes of agent concentration with time can influence the state of differentiation of cells within tissues; growth factors and transcription factors are two well-known examples of agents that function in this manner. With these examples in mind, it is natural to hypothesize that developing the capability to control the physical placement and lifetime of molecular signals is essential for tissue engineering.

Consider a simple model of a tissue engineering environment—which we call a neotissue—in which a synthetic material is combined with living cellular material; further, assume that the synthetic material is designed to create time- and space-dependent variations in concentration of an agent in the vicinity of the material (Figure 11.1a). Biomaterials science is advancing at a remarkable pace, resulting in a wide selection of technology for implantable synthetic materials [7, 8]. Within the spectrum of tissue engineering applications, this synthetic material could represent a drug delivery system intended to release growth factors into a wound bed (Figure 11.1b), a segment of a polymeric mesh intended to provide agent delivery as well as a physical support for cell attachment and migration in the regenerating tissue (Figure 11.1c), or a microfabricated prosthetic element intended to integrate in a stable fashion with cells from the host tissue (Figure 11.1d).

A spectrum of biomaterials technologies is presented in Figure 11.1; the specific requirements for delivery of an agent—that is, the identity of the molecular agent(s), its desired concentration in the tissue, and the rate of change of agent concentration with time and spatial position—will undoubtedly differ for each of these materials and every clinical application. This chapter attempts to put these technologies into perspective with regard to their potential for the delivery of molecular agents. In particular, several general questions are addressed here: What options does modern biomaterials technology offer for the controlled delivery of drugs or agents that influence the formation of tissue structure? What are the limits of agent delivery in cases where the drug must be present in time- or space-varying concentrations in order to produce the desired effect? What are the basic principles of agent transport that must guide the design and development of these delivery systems?

11.1 Technology for Controlled Delivery of Molecular Agents in Tissue Engineering

11.1.1 Controlled Release Methods in Tissue Engineering

Introduction to Controlled Release. Controlled release technology has been studied for many decades and is embodied in devices such as Norplant® and Gliadel®, which are used clinically for prevention of pregnancy and brain

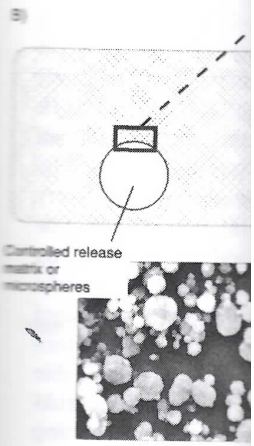

Figure 11.1. Methods f
tissue. Panel A shows th
released from a syntheti
producing a gradient of
situation can occur with
Controlled release techn
material. The inset show
(photograph from [11]).
scaffold composed of eit
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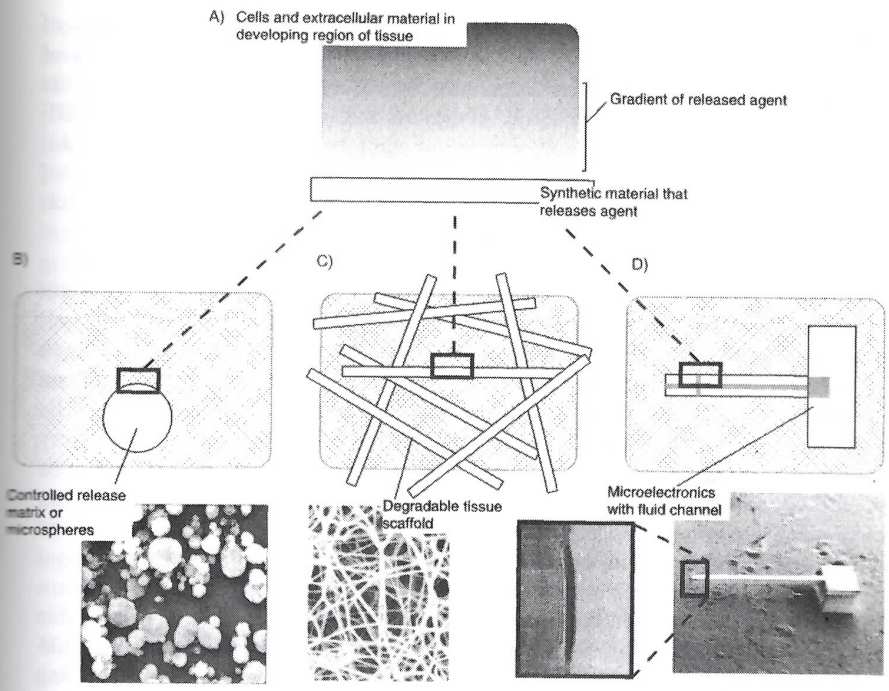


Figure 11.1. Methods for controlled delivery of agents into localized regions of tissue. Panel A shows the general situation in which an agent (indicated by white) is released from a synthetic materials into the tissue (indicated by grey/black), producing a gradient of the agent in the tissue surrounding the material. This general situation can occur with different materials, as illustrated in panels B through D. (B) Controlled release technology can provide continuous agent release from a solid material. The inset shows poly(lactide-co-glycolide) microspheres that release NGF (photograph from [11]). (C) Agent delivery by release from a degradable porous scaffold composed of either synthetic polymers or natural polymers such as collagen (shown in inset) or fibrin. (D) Agent delivery from a system with microfluidic channels. The microelectronic probe (shown in the right inset) has an internal microchannel (shown at higher magnification in the left inset), which is indicated by the grey-shaded region in the schematic illustration. In this final panel, microfabrication and photos were provided by A. Spence, S. Retterer, and M.S. Isaacson (Cornell University).

tumor therapy, respectively [9]. Controlled release has many direct applications to tissue engineering. For example, local delivery of growth factors can be accomplished by encapsulating the agent within a biocompatible polymer matrix or microsphere. The controlled-release polymer system is then implanted at the desired tissue site, where it releases the soluble factor directly into the interstitial space of the tissue (Figure 11.1b). The diffusible agent may influence survival or the function of damaged cells within the local tissue, or provide a signal that elicits cell proliferation or migration within the tissue

region. For example, controlled delivery of NGF prevents degeneration of cholinergic neurons in the brain [10, 11]. Similarly, continuous infusions of epidermal growth factor (EGF) stimulate the proliferation of neural precursors in the brains of adult animals [12], suggesting that controlled delivery of EGF may some day be useful for generating new neurons in the adult brain. Signaling molecules that are involved in development and regeneration of various tissues have been identified (some examples are listed in Table 11.1; others are described in the section that follows).

Classes of Molecular Agents that Might be Useful in Tissue Engineering. Growth factors are one class of agents that might be productively delivered to developing neo-tissues. A variety of protein growth factors that influence cell growth and differentiation have been described, and many of these factors have been tested for safety and effectiveness in human populations (see Section 11.2.1). But other classes of agents may also be good candidates for controlled delivery in tissue engineering, including agents that potentiate the immune response or modify local gene expression.

Transcription factors are transregulatory proteins that bind to specific DNA sequences (called enhancers) and modulate the efficiency and rate of transcription by interaction with the promoter. The expression of certain transcription factors is restricted to specific cell types, where they are responsible for controlling the expression of genes that characterize the state of differentiation of the cell. Transcription factors are well known for their role in patterning in the embryo and cell differentiation. In addition, the activity of some transcription factors can be regulated by hormones, such as retinoic acid and steroid hormones, which bind to the transcription factor and thereby control its biological activity. Local delivery of transcription factors, or drugs that modulate transcription factors, could provide for local control of gene expression in cells within a developing neo-tissue.

Advances in immunosuppression have greatly reduced the problem of immune rejection of transplanted organs. This improvement is primarily due to the introduction of the cyclic peptide cyclosporine, which inhibits the expression of cytokine genes in T cells (through its action on a T cell transcription factor). While cyclosporine has increased the survival of donor organs such as kidney, heart, and liver, it does not work in all cases and side effects, such as renal damage, are possible at high dose. Local delivery of cyclosporine has been successful in special cases, such as topical delivery to transplanted skin [13]. The use of controlled delivery systems for local delivery of agents that modulate the immune response may be particularly valuable in tissue engineering. Other agents that influence immune effector cells, such as antibodies against targeting selected cell populations, cytokines or other concepts that might not be prudent for systemic therapy, may be suitable for local administration. The development of local delivery methods for immunosuppression could reduce concerns regarding immunocompatibility and increase the pool of donor cells, which, in turn, could broaden the range of possibilities for effective design and implementation of tissue engineering concepts.

Controlled Release Methods. Controlled release implants are typically composed of inert, biocompatible polymers such as poly(ethylene-co-vinyl acetate) (EVAc), or biodegradable polymers, such as poly(lactide-co-glycolide) (PLGA). EVAc matrix systems have been used to release protein hormones [14], growth factors [11, 15–17], antibodies [18–21], antigens [22, 23], and DNA [24]. EVAc matrices allow a high degree of control over agent release, versatility in permitting release of a wide range of agents, and good retention of biological activity. Biodegradable polymers have also been used to release growth factors [25–27], protein hormones [28, 29], antibodies [30], antigens [31–33], and DNA [24, 34]. Biodegradable materials disappear from the implant site after protein release, which may be a significant advantage in tissue engineering applications. Polymer gels may also be useful for topical [35] or localized [36] protein delivery. Systems that release multiple protein factors are also possible [37].

Controlled growth factor delivery systems can also be used in conjunction with biocompatible matrices or porous scaffolds that provide morphological guides for assembly of regenerating tissue (Figure 11.1c). On a macroscopic level, the overall shape of the polymer scaffold provides boundaries for regrowth of the tissue. On a microscopic level, the polymeric material provides a solid framework for cell growth and tissue organization at a local site, permitting cell attachment and migration within a controllable microenvironment. This technology is described more completely in Chapter 12. A controlled delivery system can be placed in the vicinity of a polymer scaffold, allowing the creation of gradients of biological activity over the volume of the scaffold. Alternately, the materials that have been used for tissue engineering scaffolds can, in many cases, be modified to allow for controlled release of active molecular agents from the scaffold material itself.

Some of the desired characteristics of polymer scaffolds for tissue engineering have been described [2, 38, 39]. In certain cases, polymer scaffolds are sufficient to produce regeneration; for example, poly(glycolic acid) fiber meshes seeded with cultured chondrocytes produce new cartilage after transplantation [40, 41]. In other cases, tissue regeneration is more difficult to achieve and requires additional biological signals. For example, liver cells transplanted on synthetic polymer supports provide some regeneration of hepatic function, but biological function is improved when cell transplants are supplemented with hepatic growth factors (by enriching growth factors at the transplant site by surgical rearrangement of the vasculature, for example [42]).

In some cases, therefore, it might be useful to produce a polymeric material that serves multiple functions in a tissue engineering application. A multifunctional polymeric system might be designed to provide controlled release of an essential factor as well as structural support for the regenerating tissue. Recently, new approaches for multifunctional polymeric systems have been introduced in tissue engineering, including highly porous materials with embedded agents [34, 43], microparticles that are assembled with tissue progenitor cells [11], and hydrogels that are photopolymerized around cells [44]. This last technique allows cell adhesion signals, as well as linkages that are

degradable by cell-secreted enzymes, to be inserted into the polymer structure. Growth factors can be retained for longer periods by chemical immobilization. NGF [45], TGF-β [46], and EGF [47] possess biological activity when chemically immobilized to a surface.

Recently, methods for surface immobilization have been developed that result in gradients of the bound agent (Figure 11.2). Alternatively, the growth factor can be modified by conjugation to a water-soluble carrier, which prolongs its retention at a local site [26, 48–50]. Methods for producing materials that release the immobilized factor in response to a trigger, such as enzymatic activity released by a migrating cell, have also been demonstrated [51, 52].

11.1.2 Microfluidic Systems for Controlled Release and Tissue Assembly

Microfluidics and Microfluidic Devices. Microfluidics is the development of miniaturized devices for handling fluids. Microfluidic devices are composed of networks of interconnected channels, pumps, valves, mixers, separators, and a variety of other components—all on the scale of microns or less. Microfluidic systems have many potential applications including chemical analysis, microchemical reactors, combinatorial chemistry, DNA and protein separations and analysis, and cell sorting. These diverse applications reflect the many advantages in processing fluid systems on small dimensions [53–55]. Precise volumes of fluid can be moved rapidly and efficiently. Chemical analysis is especially accurate owing to the combination of small sample volumes and sensitive detection methods. Diagnostic and delivery methods

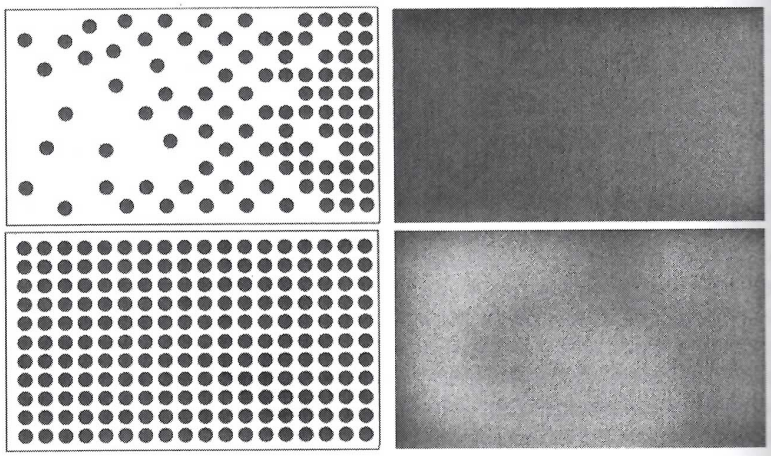


Figure 11.2. Surface gradients of agents: controlled presentation of molecules attached to a surface by immobilization in either uniform (bottom) or right-to-left gradient (top). In these photos, a fluorescently labeled protein was immobilized to a synthetic polymer hydrogel. Images of agent on the hydrogel *in vitro* were collected via epifluorescence microscopy [Gemeinhart and Saltzman, unpublished data].

often can be integrated in a single device. Microfluidic devices have low power requirements, and certain devices can be made suitable for implanting into tissue. Techniques for fabricating microfluidic circuits include reactive-ion etching, lithography, embossing, casting, and molding.

Microfluidic devices require a mechanism for pumping the fluid, in a controlled fashion, through the microscopic mechanical elements. A variety of pumping methods are available, including electro-osmotic pumping for ionic fluids, positive displacement pumps using piezoelectric components, pneumatic pumps, and bubble or surface-tension pumps that rely on moving gas–liquid interfaces to displace fluids. Flow rates up to 100 μL/min have been achieved in an electro-osmotic flow, although a significant voltage must be applied across the channel to achieve high flow rates [55, 56]. In addition, electrokinetic flows can be compromised by contamination on the channel walls and ohmic heating. Some of the same mechanisms that are used to pump fluids can be exploited to construct valves that regulate the flow in microfluidic circuits [57]. Thus, a variety of passive and externally actuated valves, using piezoelectric, electromagnetic, or pneumatic control, have been designed, including valves that rely on controlling the position of a bubble or a gas–liquid interface in a microchannel [53].

Microfluidic Systems for Cell Patterning. How can microfluidic systems be useful in developing new tools for drug delivery and tissue engineering? One area of application is the induction of pattern formation in cell cultures by microfluidic control of cell positioning. The use of microfabrication techniques to pattern cells on surfaces was demonstrated more than a decade ago [58], and a recent review summarizes developments since then [59]. The underlying idea in many applications is to use microfluidic channels to deliver precise amounts of cell-binding agents, usually proteins, to specific locations on a substrate. The agent is adsorbed or otherwise immobilized only on the part of the substrate that is in contact with the fluid. Subsequently, cells that bind to the immobilized agent can be introduced through the same microchannels that were used to deliver the agents to the substrate, which leads to the formation of specific patterns of cell deposition.

These principles of microfluidics can be used to develop intricate arrangements of multiple cell types on a substrate by introducing agents that differ in cell adhesion selectivity within individual microchannels. Subtle arrangements are possible because of an intrinsic property of microfluidic flows: when fluid streams are combined in microchannels, the combined streams show no inertially induced instabilities that could disrupt the boundary between them. Instead, the combined streams flow in parallel down the microchannel with only diffusional exchange, which is a relatively slow process. This characteristic of low Reynolds number flow can be used to create spatial patterns of agent adsorption—and therefore cell adhesion—within single microchannels. For example, if one of the streams contains an adhesive glycoprotein that adsorbs to the material, the glycoprotein adsorbs nonspecifically on the part of the channel in contact with that particular stream [60]. Multiple fluid

streams can be combined into a single channel with the glycoprotein present in every other stream. As a result, the surface of the channel exhibits stripes of glycoprotein adsorption. If cells that bind to the glycoprotein are then introduced into the channel, they will bind along the glycoprotein stripes. By selecting the microchannel geometry and controlling the relative flow rates of various streams, a variety of cell culture patterns can be formed inside a microchannel.

These basic techniques are building blocks that can be used to fabricate more complicated cell culture patterns. For example, by combining several microfluidic layers that contain spatial variations in their surface properties, three-dimensional cell culture patterns composed of multiple types of cells can be formed [61]. These three-dimensional patterning techniques have been used to place cancer cells in close proximity with endothelial cells, resulting in spatial arrangements that could be used to mimic blood vessel formation in tumors. Microfluidic systems may also prove useful in patterning cells that are not attached to a solid substrate. As a first step, a microfluidic system has been used to form intricate, stable, three-dimensional patterns of freely suspended vesicles under controlled conditions [62].

Microfluidic Systems for Local Agent Delivery. A second application of microfluidics in tissue engineering is even more closely related to drug delivery: the use of microfabricated prostheses to deliver chemical agents to precisely defined regions of tissue or even into the interior of cells. Arrays of silicon microprobes and microneedles have been fabricated using anisotropic etching [63]. The probes have the shape of a pyramid and range in height from a few to several hundred micrometers. The tips of the probes are smaller than 0.1 μm, which means they can easily penetrate the cell membrane either to deliver an agent through the probe tip or to provide access for intracellular measurements and analysis. The microarrays have been used to deliver a plasmid vector containing a gene for β-glucuronidase to tobacco cells, which soon after showed a transient expression of β-glucuronidase. Similar techniques have been used to fabricate arrays of hollow microcapillaries [64]. Because the lumen diameter of the microcapillaries is known precisely and does not vary along the capillary length, more precise control of the volume of fluid delivered to cells is possible. However, the microcapillaries do not have a sharp tip, and therefore they may not be able to penetrate smaller targets such as bacterial cells or viruses.

Individual microchannel probes have also been developed to deliver precise amounts of an agent to localized regions of tissue. For example, a silicon-based probe for delivery to neural tissue consists of several parallel microchannels that are housed in a flat shank about 70 μm wide and 4 mm in length [65]. Some channels can be used to deliver various agents to the tissue, while other channels are used to measure electrical signals from the neurons. The probes have been used to modulate the neuronal activity of guinea pig brain tissue by sequentially injecting solutions of kainic acid, a neural stimulant, and γ-aminobutyric acid, a neural depressant [65].

Similar microfabrication techniques have been used to construct microneedles and microneedle arrays for intravenous injection [66, 67]. The microneedles have a rectangular cross-section with typical dimensions of 20 × 40 μm and a wall thickness of about 20 μm. An array of 25 such microneedles can deliver flow rates ranging from 50 to 650 μL/min. Microneedles containing multiple outlet ports have also been fabricated. Although it has yet to be demonstrated, the microneedle arrays could provide a means for sustained transdermal drug delivery.

Microfabrication techniques have also been used to construct controlled release microchips [68]. The microchips contain an array of small reservoirs that are filled with various agents to be delivered to the surrounding tissue. The opening of each reservoir is sealed with a material that can be selectively dissolved by an electrochemical reaction. One embodiment of the process uses reservoirs that are sealed with a thin gold membrane that also acts as the anode in an electrochemical reaction. When release of the compound from a specific reservoir is desired, a potential of about 1 V is applied to the membrane to cause it to dissolve, which allows the agent to diffuse out of the reservoir. A variety of drugs, including liquids, solids, and gels, can be delivered from the microchip in a controlled and sustained release. The amount of drug in each reservoir can be adjusted arbitrarily, and the release rate can be programmed or controlled remotely, which could enable precise control of delivery rates to match particular therapeutic requirements.

11.2 Controlled Release of Agents in Time and Space

11.2.1 Tissue Regeneration is a Step-wise Process Involving Timed Introduction of Molecular Agents

Developmental Signals and Tissue Engineering. Natural processes of development and regeneration occur in a sequence of steps; molecular signals and cellular responses unfold in time through a coordinated sequence of spatial changes. The step-wise nature of development is long known. Recent studies are revealing a complex relationship between secreted signals and cell fate; for example, gradients of FGF-8 influence the cytoarchitectonic map of the cortex [69], and gradients of BDNF and NT-3 change with time to control the spatial pattern of innervation in the cochlea [70]. In adults, the step-wise mechanisms of regeneration are being revealed in studies of angiogenesis [71] and wound healing [3]; carefully orchestrated patterns of soluble bioactive agents are observed in both cases.

In tissue engineering, the transformation of a neotissue into a functional replacement tissue also occurs in a series of steps, in which each step potentially involves different biological signals. The step-wise pattern of transformation from a neotissue into a functioning tissue has been documented in many studies including tissue-engineered skin, nerve, and heart valves (Table 11.1).

Clinical Experience with Molecular Agents of Interest in Tissue Engineering. Although several individual agents that seemed promising—such as VEGF for angiogenesis and PDGF for wound healing enhancement—have been tested in humans, the clinical experience so far is disappointing. Platelet-derived growth factor (PDGF), for example, is a component of an FDA-approved product for healing of diabetic ulcers; the topically applied gel is manufactured by Ortho-McNeil from recombinant PDGF that is produced by Chiron. Many other protein growth factors have been tested for effectiveness in therapy. Fibroblast growth factor (bFGF) has been intravenously administrated to patients in the first 12 hours after thromboembolic stroke; although initial clinical trials were promising, the phase 2/3 trial was halted after 300 patients were tested, because of adverse side effects. NGF has been tested in a variety of clinical applications including systemic administration of the protein and local gene therapy to produce NGF in the brain. Subcutaneous administration of recombinant human NGF, produced by Genentech, reduced the sensory neuropathy associated with HIV infection in some patients [72], but early results were not considered promising enough to earn FDA approval. In a recent preliminary clinical study, topical application of NGF (10 μg/eye, 6 to 10 times daily) led to healing of corneal neurotrophic ulcers with few side effects [73], although this local therapy has not yet been through extensive trials.

Angiogenic factors, or factors that stimulate the formation of new blood vessels, are among the first tissue engineering agents to be tested in humans. Two such agents were championed by two of the most successful biotechnology companies in the U.S.: Genentech (VEGF) and Chiron (FGF-2). Although the production and pre-clinical testing of these compounds represent milestones in the development of protein therapies, and have led to advances in the understanding of angiogenic mechanisms in animals, both agents were unsuccessful in phase 2 clinical trials.

At present, it is difficult to determine whether the failure of these particular agents in clinical trials is due to a problem with the mechanism of action of the agents in humans or a failure to deliver the agent in the correct dose at the target tissue for the appropriate length of time. A definitive test for this new class of therapeutic compounds requires a reliable and controllable technique for the precise control of agent delivery and localization. Providing this level of control over local agent delivery could be the most important initial contribution of controlled release and microfluidics in tissue engineering. In retrospect this failure is easily rationalized, considering the vast difference between administration of single agents (even powerful ones) and the deliberate temporal and spatial coordination of chemical signals that occurs during regeneration.

11.2.2 Controlled Release Materials, Microfluidics, and Temporal Control of Agent Delivery. One major advantage of drug delivery methods in tissue engineering is the potential to control the spatial or temporal pattern of agent delivery. By localizing the source of agent release in the neotissue, gra-

dients of biological activity can be produced within the microenvironment; stable gradients of growth factors and other proteins are maintained near the surface of polymers immersed in unstirred fluids or embedded in collagen gels [74, 75]. It has long been speculated that gradients of soluble factors, called morphogens, can influence pattern formation and tissue development in embryos (see Chapter 3). Interestingly, the gradients produced by controlled release polymers are similar to signaling gradients measured in embryos. Gradients of biological activity can be maintained in animal tissues for several weeks by a single administration of a controlled release polymer [76] (Figure 11.3). By utilizing controlled release, the biological effects of potent signaling molecules are confined to the tissue immediately surrounding (usually within ~1 mm) the implant [77].

Many controlled release systems rely on dissolution of solid material or partitioning from a non-aqueous phase as an element of release. High concentrations of agent can therefore, be delivered to the tissue. In the case of NGF (Figure 11.3), tissue doses of greater than 30,000 ng/mL were achieved; this concentration is ~10,000 times higher than the concentration of NGF that is active in tissue culture. Once the agent is released from the material, transport deeper into the tissue depends on properties of the agent (for example, its size, solubility, and stability) and properties of the local tissue space (for example, its cellular composition, vascularity, rates of interstitial flow). The dynamics of agent release from a controlled delivery device and local transport in the tissue have been most thoroughly studied in the brain [78], although the basic prin-

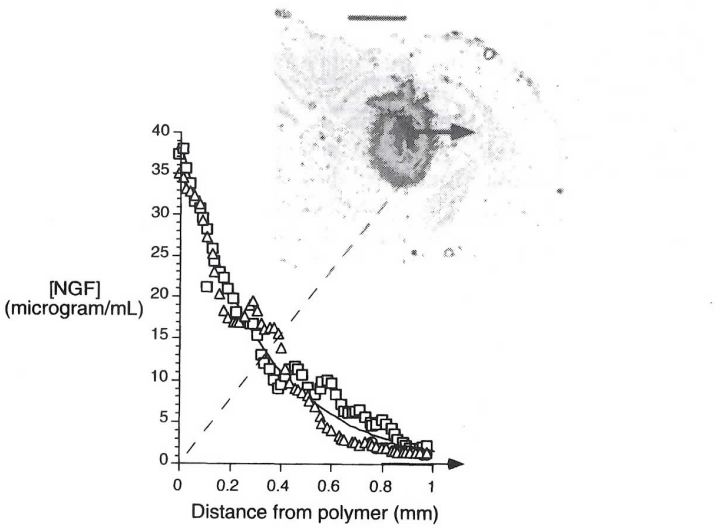


Figure 11.3. Concentration gradients produced by controlled release systems, surrounding a controlled release polymer matrix (photo and graph of gradients adapted from Krewson and Saltzman [92]).

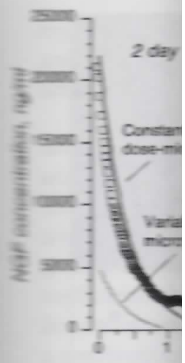
ciples appear to apply to other tissues, even tissues with markedly different architectures such as the reproductive tissue [79].

Controlled release from polymeric materials has some important limitations for sustained delivery to a local site. Because most controlled release systems depend on diffusion of agent through the material during release, the rate of agent release typically decreases with time. When the system is used for local delivery to a tissue, the decreasing rate of agent release is reflected in a decreasing concentration of agent in the tissue adjacent to the material (Figure 11.4). An important advantage of microfluidic delivery systems is the potential to control the rate of agent delivery to the tissue continuously with time, which would allow for temporal control of the peak concentration, that is, the concentration in the tissue immediately adjacent to the delivery system. Provided that only small volumes of fluid are introduced with the agent, the dynamics of agent transport in the tissue are independent of the delivery system; therefore, microfluidic systems will also lead to agent gradients in the local tissue. But, by controlling the rate of agent delivery, a microfluidic device can (in principle) be used to produce patterns of agent concentration that are programmable with time (Figure 11.4).

11.2.3 Control of Spatial Gradients using Controlled Release Materials and Microfluidics. When agents are delivered by controlled release materials into the brain, the local movement of molecules is determined by the balance between agent diffusion, in which molecules move away from the material by transport down a concentration gradient, and clearance, in which molecules are removed from the transport pathway by degradation, metabolism, binding, or partitioning into capillaries. This balance of diffusion and clearance leads to the development of stable gradients of agent in the tissue near the implanted material (Figures 11.3 and 11.4).

The size of the treated region of tissue (that is, the region that is exposed to concentrations within the biologically active range) is determined by the relative rates of diffusion and clearance. For most agents—including proteins, small drugs, and even virus-sized particulates—the relative rates of diffusion and clearance produce a region of treatment that is approximately 1 mm in size (Figure 11.4). Models of the local transport of molecular agents can be used to examine mechanisms of agent migration and to predict the effect of changes in system variables (such as agent size and clearance rate) on the size of the treated region of tissue. The mathematical and physical principles for modeling agent transport are reviewed in Appendix B. One example of the use of these principles has already been outlined in Chapter 3 (see Equations 3-1 to 3-3 and the related discussion); other examples are provided in the exercises at the end of this chapter.

In many cases this limited range of agent penetration may be beneficial, since highly potent compounds must be confined to specific anatomic regions. However, in other cases it may be desirable to deliver an agent over larger regions; for example, regions of ~1 cm in diameter correspond roughly to the size of neuroanatomical nuclei in the human brain. One simple method for



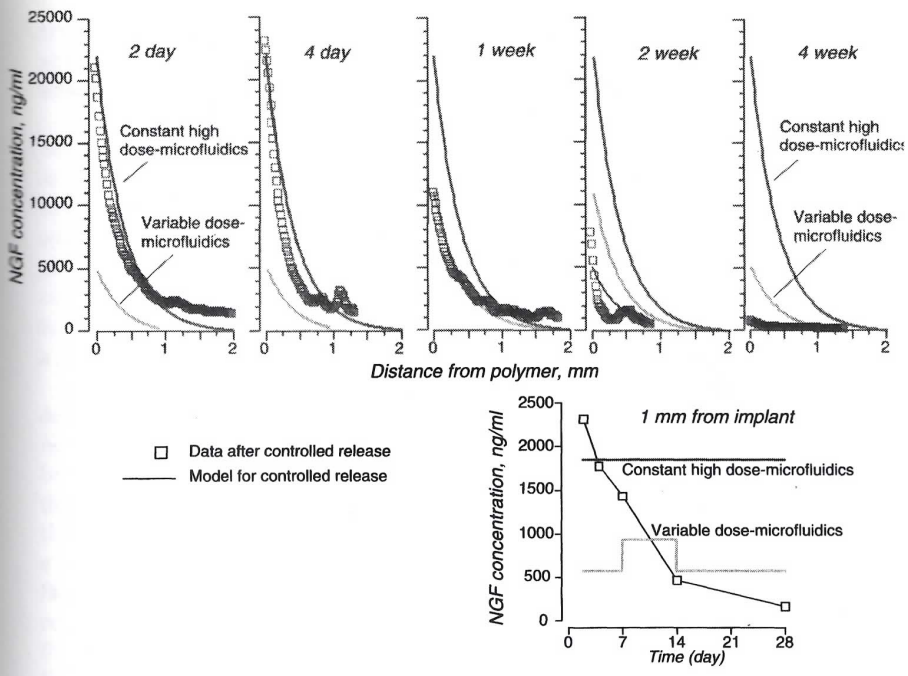


Figure 11.4. Dynamics of NGF delivery to a localized region of tissue. This figure shows NGF concentrations in the tissue surrounding an implanted controlled release matrix (see Figure 11.3). Measured concentrations in the tissue (squares) are dramatically elevated for several weeks after implantation. The fall of concentration with distance from the implant is due to a balance between agent diffusion (down the gradient, into the tissue) and agent disappearance from the tissue (by metabolism or clearance); mathematical models can be used to predict the tissue spatial profiles and their rate of change with time [93], as shown by the thin black lines. In principle, microfluidic drug delivery allows the option of controlling the rate of agent delivery to the tissue over time and therefore could be used to maintain a continuously high concentration or a variable concentration. The graph at the bottom indicates the concentration of agent that would be experienced by a cell 1 mm from the implant surface in each of the delivery scenarios.

dispersing active protein over a larger volume would require controlled placement of a suspension of cells or microspheres into the brain interstitium. Mathematical models of agent transport have been used to predict agent concentrations following delivery from polymer matrices and microspheres (Figure 11.5; details of the calculations have appeared elsewhere [80], but these calculations are similar to the ones presented in Chapter 3). Computer-generated images—in which colors correspond to ranges of concentration—provide a useful means for comparing agent concentration profiles following release from both a polymer matrix and uniformly distributed polymeric microspheres. When small spheres are spaced less than 1 mm apart, larger regions

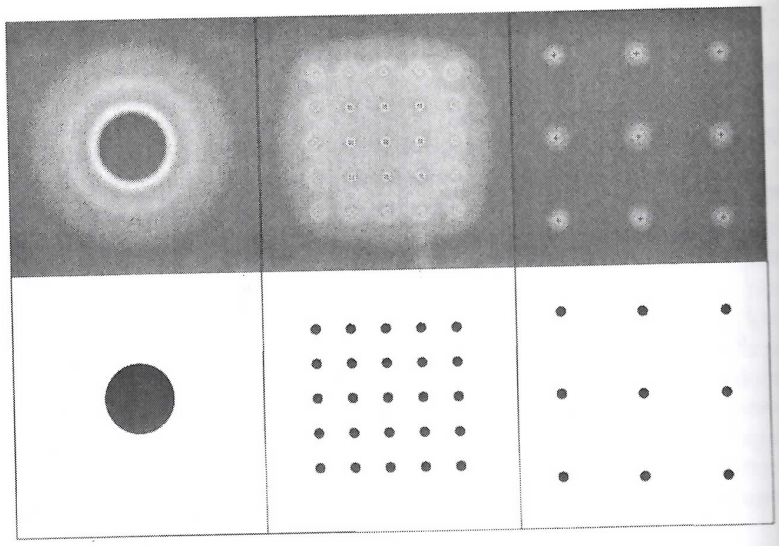
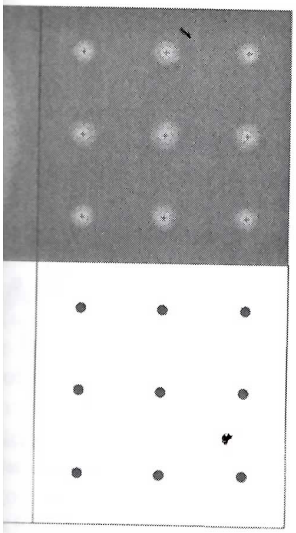


Figure 11.5. Delivery by arrays of controlled release systems. The figure shows concentration gradients surrounding large matrices (~1 mm in diameter) or arrays of smaller microspheres (~10 μm in diameter arranged with close, 1 mm, or distant spacing), adapted from computer-generated predictions as described by Mahoney and Saltzman [80].

of high agent concentration are visible. When microspheres are placed greater than 2.5 mm apart, they essentially behave as isolated point sources distributed throughout the tissue. In this configuration, the volume of tissue exposed to therapeutic agent concentration is small. This method of computer simulation (based on the experimentally measured characteristics of agent transport, as in Figure 11.3 for the case of NGF) can be coupled with radiological studies to guide the placement of ensembles of controlled release systems in a tissue to tailor agent distribution to the anatomy of a tissue region or disease process. In tissue engineering, this pharmacotectonic approach to agent delivery can be used to localize regenerative agents within irregularly shaped regions of tissue or neotissue.

In other cases it may be advantageous to deliver agents to tissue in such a way that they spread over distances that are much larger than the penetration distance shown in Figure 11.5. One possibility is to deliver fluids by pumping them directly into tissue through a microneedle or other prosthetic device. In this case, the transport of the agent in the tissue is enhanced by convection, which may be capable of carrying the agent further than the diffusion penetration distance. The diffusion penetration distance is governed by the rate of diffusion modified by the rate of agent clearance. Diffusion rates are determined by the physicochemical properties of the agent and tissue, and are independent of the delivery scheme. In convection-based deliv-



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ery, the penetration distance is determined by the relative rates of convection and elimination, and convection rates can be determined by the delivery scheme.

The velocity of fluid in tissue under constant infusion through a microneedle is determined by a variety of factors, including the structure of the tissue and the interstitial matrix and fluid transport across microvascular membranes and into lymphatics, if present. A simple model views convection in the interstitial space as flow through a porous medium governed by Darcy's law, which gives a linear relationship between flow rate and the pressure gradient with coefficients that reflect the extracellular volume fraction and the tissue hydraulic conductivity. If an agent is dissolved in the fluid, its transport is governed by a combination of convection, diffusion, and clearance.

The relative importance of convection in comparison with diffusion is determined by the Peclet number. For example, calculations for the continuous infusion of a 180 kDa protein at a rate of 3.5 μL/min through a needle in the brain yield a Peclet number that exceeds 10 at a distance of almost 2 cm from the injection point [81]. Thus, diffusion is unimportant over this distance, and the injected protein is predicted to advance outward from the injection point in a sharp front. After 12 hr of continuous infusion, the front is located 1.5 cm from the injection point with little loss in protein concentration at the leading edge. The front will continue to advance as long as infusion continues, but the fluid velocity decreases with increasing distance from the injection point. Eventually, the velocity will decrease to the point that diffusion becomes significant.

Delivering compounds continuously and promoting their spread over relatively large distances (~1 cm) is important in delivering anti-cancer agents to brain tumors. However, in tissue engineering applications, it may be important to deliver smaller volumes of potent morphogens or other growth agents at specified locations [82, 83]. Furthermore, since cell behavior in the neotissue can be sensitive to concentration gradients, it may be desirable to control concentration profiles in order to stimulate directional or spatial changes. In this event, it may be possible to combine microfluidic systems with convection-enhanced delivery to control the location and profile of agents at distant locations from the injection point (Figure 11.6). Microfluidic circuits can be designed to deliver flow rates and outlet concentrations of agents that vary in time in prescribed ways. For example, if a discrete bolus of an agent is infused through a microneedle, it will spread through the tissue radially as a spherical shell whose thickness depends on the volume of the bolus and the distance from the injection point (Figure 11.7). The location of the shell can be controlled by adjusting the infusion rate as the shell advances. With only a single infusion point, the spherical symmetry of the shell will be preserved as it advances, at least in homogeneous tissue. However, if the agent is delivered by infusion at a series of spatial locations (as in Figure 11.6), then more complex shapes can be formed by allowing the shells from the delivery points to overlap with each other. Furthermore, if the concentrations of the agent differ among the various infusion points, then concentration gradients can be estab-

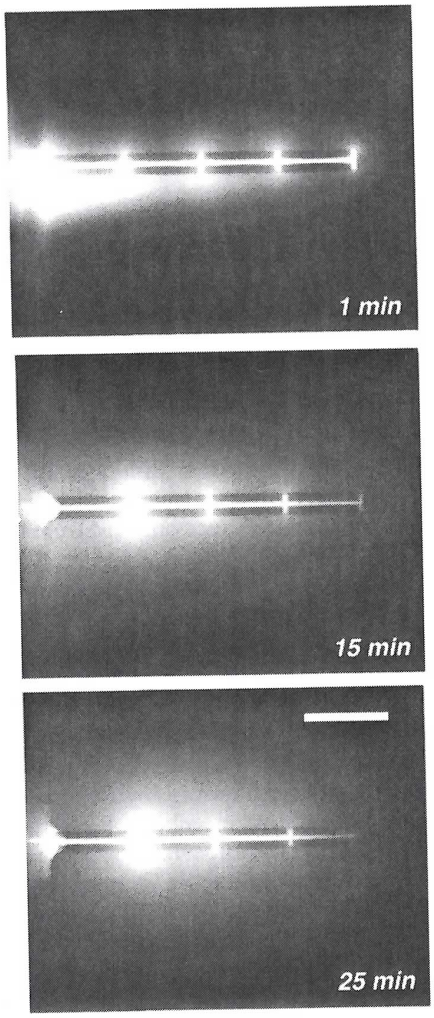

Figure 11.6. Flow in a microfluidic channel. The figures show flow and delivery of 3 kDa dextran through a microfluidic channel at 1, 15, and 25 min after introduction of dextran to the reservoir (left side of photo). The microfluidic device was implanted within an agarose gel; diffusion of dextran from the channel into the surrounding gel can be observed. Scale bar = 500 μm. Figure provided by Scott Retterer.

lished within the overlapping shells far from the injection points. Of course, clearance of the agent and diffusion would eventually degrade the concentration profile that is established at the end of infusion, but the infusion cycle could be repeated to reestablish the desired profile.

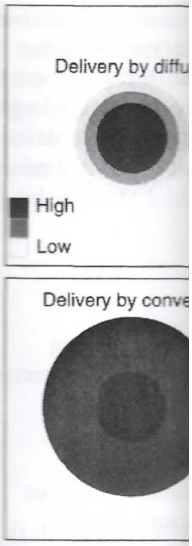

Figure 11.7. Local spr
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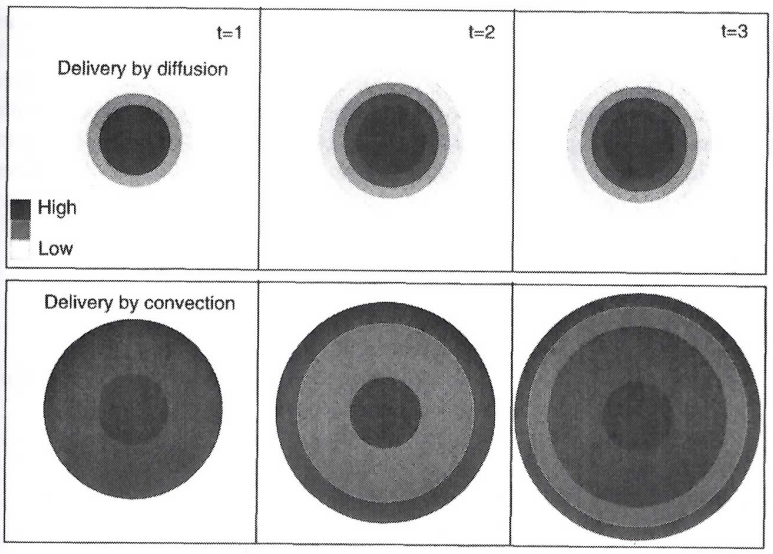

Figure 11.7. Local spread of agents predicted by models of diffusion and convection. Local spread by diffusion is shown in the top three panels. In this case, concentration gradients develop within the tissue near the device and remain stable until the agent is completely released (compare to Figure 11.4). Local spread of agents can be enhanced by convection, as illustrated in the bottom three panels. Convection can enhance the penetration distance, as shown in the left panel, but it can also allow for programmed changes in infusion concentration with time. The change in color of the outermost red region in the panel F reflects degradation of the agent with time. The delivery vehicle is shown in black; concentration ranges are indicated by shades of grey.

11.3 Future Applications of Controlled Delivery in Tissue Engineering

Controlled release is a well-established approach in drug delivery. However, the combination of controlled release technology and microfluidics is likely to produce new delivery methods for a variety of biological agents (including drugs, proteins, and genes) that are essential in tissue engineering. Microfluidics not only provides new tools for fabricating controlled release platforms, but also provides a new method for delivering agents directly to tissue. The essential feature of delivery with microfluidics is that diffusion can be combined with convection to offer greater versatility in design than is available with controlled release alone. In particular, the penetration distance of an agent in tissue can be increased by using convection-based delivery, and if flow rates, concentrations, and release sequences can be controlled using microfluidics, then spatial patterns of agents could be established to direct tissue growth and other cellular responses.

Unlike controlled release, which has been tested extensively both in animals and in humans, microfluidic methods have not been challenged by

long-term use in a tissue environment. Furthermore, the materials that are frequently used to fabricate laboratory microfluidic prototypes may not be appropriate for clinical applications. The ultimate clinical impact of microfluidics on tissue engineering may depend not only on our understanding of the physics underlying the delivery method, but also on our ability to develop compatible microfluidic devices. In any event, it is clear that controlled release and microfluidics are key methods in tissue engineering.

Summary

- The engineering of many tissue structures—such as the branching architectures found in many tissues or the intricate network architecture of the nervous system—will require new methods for producing controlled spatial and temporal gradients of agents in developing tissues.
- Controlled release technology has many direct applications to tissue engineering; for example, local delivery of growth factors can be accomplished by encapsulating the agent within a biocompatible polymer matrix or microsphere.
- Controlled growth factor delivery systems can also be used in conjunction with biocompatible matrices or porous scaffolds that provide morphological guides for assembly of regenerating tissue within a controllable microenvironment.
- Microfluidic devices, which are composed of networks of interconnected channels, pumps, valves, mixers, and separators, will be useful in developing new tools for drug delivery and tissue engineering, including microfluidic control of cell positioning and local agent delivery.
- In tissue engineering, the transformation of a neotissue into a functional replacement tissue occurs in a series of steps, in which each step potentially involves different biological signals that can be introduced by controlled delivery methods.
- Modeling of the dynamics of agent diffusion and convection at local tissue sites will be an essential tool for developing new delivery systems that provide spatial and temporal control over agent delivery.

This chapter was revised and expanded from an original manuscript, co-authored by W.M. Saltzman and W.L. Olbricht, that appeared previously [1].

Exercises

Exercise 11.1

Ethylene–vinyl acetate copolymer discs are prepared, containing dispersed particles of BSA and nerve growth factor (particle composition BSA:NGF 2500:1 by weight). The matrices contain 40% particles (by weight) and the particles