Clinical Implications of Cell Function in Bone Grafting

C. Andrew L. Bassett, M.D.*

Most orthopedists are familiar with the smallest operative detail of a given bone grafting procedure. Few, however, are aware of the principles of cell behavior which may determine the success or failure of the procedure. We quickly recognize the radiographic features of a hypertrophied, atrophied, resorbed, or sequestered bone graft, yet rarely do we appreciate the cellular and environmental factors responsible for these results! For example, why was the dead, cortical, bone graft, illustrated in Fig. 1A–B incorporated so well, while the dead cortical graft shown in Fig. 2A–B was sequestered?

Cell action is required to unite a bone graft with the host, to increase or decrease the mass of the graft, and to precipitate rejection. This being the case, it is essential first to identify the origin of cells involved in the evolution of a bone graft and to define, as precisely as possible, their character. During the first half of this century, extensive discussions centered on the recipient site, vis-a-vis the graft itself as the primary source of bone-forming cells. Many investigators claimed that, following grafting, osteogenesis was solely the result of cells from the host bed and doubted that cells in the graft survived even after autotransplantation. Much of the confusion resulted from a failure to differentiate between cortical and cancellous bone and between mature and embryonic grafts. Experiments in the past 15 years, however, have demonstrated that surface cells in free, mature, cancellous bone grafts can survive transplantation, if properly handled, and will participate in the total early osteogenic response around the graft.[10, 15, 41] On the other hand, it is doubtful whether significant numbers of osteocytes persist in a graft of mature bone, whether cortical or cancellous. Even if they do survive, it is unlikely that they can free themselves from their lacunar entrapment to join other cells in the "pool" surrounding the graft. The extracellular bony matrix surrounding these osteocytes is, of course, "dead" and, therefore, passive. It may serve a mechanical function to occupy space and provide surfaces for cell migration. Furthermore, it imposes a micro-anatomic form to guide cells during reorganization, serves structurally in resisting applied forces and thereby generates electric potentials. When incorporated by the host, this dead bony matrix usually is removed to a large extent. If put to mechanical use during replacement, the newly deposited extracellular matrix assumes a new orientation. Since the cells responsible for this reorganization come primarily from invading host vessels, it is reasonable to ask whether the survival of surface cells has any practical significance. The answer is very much in the affirmative!

From the Orthopaedic Research Laboratories, Columbia University College of Physicians and Surgeons and the New York Orthopaedic Hospital, Columbia-Presbyterian Medical Center, New York, New York.

* Career Scientist, Health Research Council of the City of New York.

Reprints: 630 West 168th Street, New York, New York 10032.

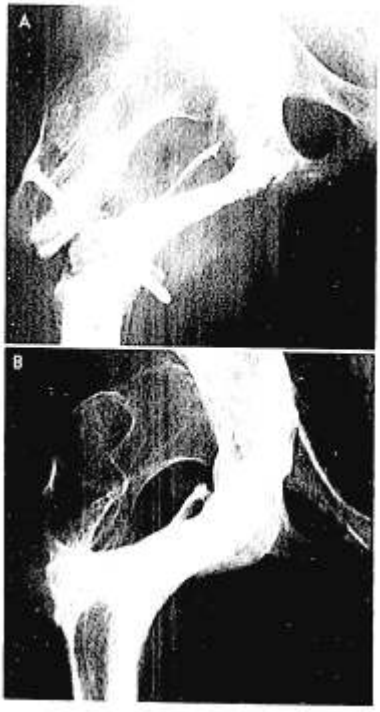

FIG. 1 A–B. A, 12-year-old boy, 5 months post-ischiofemoral arthrodesis, using frozen, tibial cortical homograft (allograft). Note uniting callus at femoral and ischial contacts. B. Two years postoperative. Note marked increase in bulk of graft under the stimulus of functional forces.

Vessels can invade an osseous graft only when shearing motion between compliant soft tissues and non-compliant hard tissues or between two non-compliant, hard surfaces is largely eliminated. Elimination of this motion frequently is the function of callus, which is elastic in its initial phases and, subsequently, becomes progressively stiffer. It is important, therefore, to establish the largest possible mass of callus in the shortest possible time in order to "glue" the tissue and graft (or grafts) firmly together. This process is accomplished most effectively by osteogenic cells. Therefore, the more *viable* cells of this type, the better! Although the major source of these cells is in the more vascular regions of both soft and hard tissues at the recipient site, an appreciable number of osteogenic cells can be transplanted successfully on properly prepared cancellous grafts. In spine fusions (particularly in the lumbosacral region), where motion cannot be eliminated, this source of additional cells can spell the difference between union and pseudarthrosis.

What are the relative cellular contributions of the recipient site and the graft during the course of graft incorporation? First, the graft and host must be united by a bridge of callus or reactive new bone.[22, 24] This essential step is accomplished equally well by cells from the graft or host as noted in the paragraph above. The bridge eliminates or reduces shearing motion at the interface between the transplant and its bed, thereby facilitating penetration of host vessels and

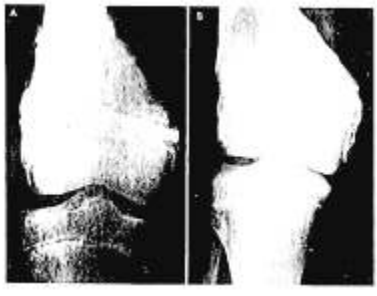

FIG. 2 A–B. A, 13-year-old boy with Salter Type II injury of the distal femoral epiphysis, fixed 3 months previously with two "dowel" grafts. The distal graft was aged ox bone, sterilized by autoclaving; the proximal graft was a fresh autologous fibula. B, Four years after operation, the proximal autologous cortical graft is completely remodeled, while the denatured heterograft (xenograft) still is visible as a "sequestrum." Note radiolucent zone around graft.

making a mechanically sound host–graft unit. The second step, vascular invasion, is a product primarily of the host and is responsible for an influx of cells which will sweep toward the center of the graft. These invading host perivascular cells and vessels set the stage for the third and fourth steps, namely, a sequential removal of pre-existing bone matrix and its replacement by new bone. This process is illustrated in Figures 3, 4 and 5.

Inherent in the foregoing discussion is the implication that both surface cells on spongy bone and perivascular connective tissue cells in the bed are capable of forming bone. In fact, these mesenchymal-stem elements can be considered to form a "pool" of unspecialized cells[12] which are capable of responding to host needs in a variety of ways. They can specialize as osteoblasts, osteoclasts, chondroblasts, chondroclasts, fibroblasts and, on occasion, as lipoblasts, depending upon nutritional factors and stimuli in their microenvironment.[3, 5, 27] Furthermore, specialization does not preclude a return to the pool under appropriate conditions. In view of this broad potential, it seems proper to label unspecialized cells in the pool only after they have "committed" themselves and begun to produce the organic precusors of cartilage bone or fibrous tissue. The use of terms such as "osteoprogenitor cell" and "preosteoblast" for cells still in the pool may be excessively restrictive, since they could just as well be called "prechondroblasts," "chondroprogenitor cells," etc.

Bone formation has three basic requirements: a proper cell, proper nutrition, and a proper stimulus. If these three factors do not co-exist, osteogenesis can not occur. What then is a proper cell? Basically, as noted in the previous paragraph, it is a mesenchymal stem cell which, in the adult, generally arises from perivascular cells associated with proliferating blood vessels, reticular elements of bone marrow, trabecular surfaces, and the cambium layer of the periosteum. Trueta[37] has proposed that

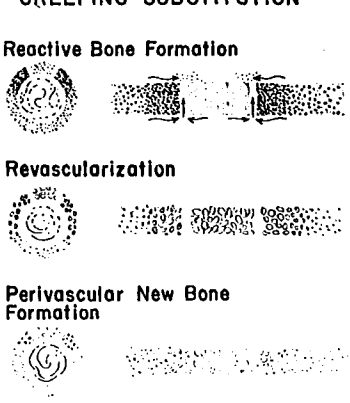

FIG. 3. Scheme of the major phases of bone graft incorporation. In the first phase (reactive new bone formation) callus can arise both in the medullary canal and periosteum to bridge the gap between graft and host. During the second phase, revascularization, the graft is mechanically weakened by osteoporosis. If subjected to excessive forces at this age, it may fail.

the endothelial cell also is osteogenic. The majority of evidence, however, suggests that this cell must leave its habitus and alter its cellular "equipment" before it can enter the perivascular pool and emerge as an osteoblast. It seems clear that these mesenchymal cells contain operative genes which direct the production of the collagen and proteinpolysaccharides of bone, if the proper raw materials and stimuli are supplied.

ENVIRONMENT AND CELL SPECIALIZATION

Many factors will determine the availability of nutrients. Some of the more important of these are: (1) the nutritional status of the total organism, (2) the distance of the cell from its source of supply in the adjacent vessel, (3) the diffusion rate in the extracellular space (determined, in part, by fixed charge and filtrational characteristics

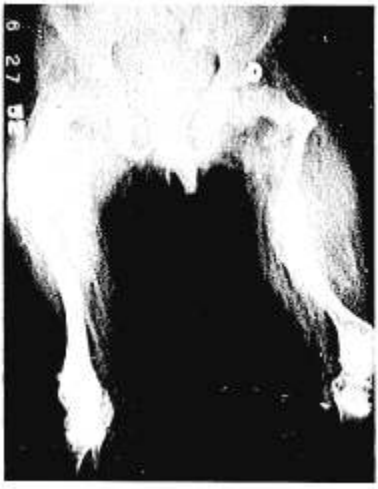

FIG. 4. Nine-year-old boy with osteogenesis imperfecta, numerous femoral fractures, malunion and pseudarthrosis.

of the matrix, (4) the interposition of barriers to diffusion (e.g., other cells) between the cell and its source of nutrition, (5) the propulsion of extracellular fluids by cyclic deformation of tissues, (6) the electrophoretic and electro-osmotic "pumping" action arising from stress-generated electric potentials.[4] Cell nutrition is dependent not only on these extracellular factors, but also on properties of the cell itself. These properties involve the movement, fixed-charge characteristics and pore size of the plasma membrane, and the rate of pinocytosis and intracellular streaming.

A significant amount of evidence supports the view that specialization of mesenchymal cells can be determined by external factors, both in vitro and in vivo. A summary of the details of several studies will be used to demonstrate this point. Endosteal and reticular elements (mesenchymal cells) arising from explants of embryonal bone produce bone, cartilage, or fibrous tissue or a combination, depending upon the conditions present in a tissue culture system.[2, 3] When cells are allowed to become compacted, through repeated excision of the zone of outgrowth and given adequate amounts of oxygen (35% O_2 in the gas phase), bone formation results. The bone has histologic and ultrastructural characteristics of fiber bone and is heavily but not uniformly calcified. These same cells, permitted to compact and subjected to low oxygen concentrations (5% O_2 or lower in the gas phase), produce only cartilage. If the zone of outgrowth is stretched repeatedly, so compaction is not permitted, and adequate amounts of oxygen are present, highly oriented fibrous tissue, resembling young tendon or fascia is obtained. From these and other studies,[20, 27] it is clear that both nutritional (O_2) and physical factors (tension vs compaction/compression) determine, in part, the pathway of cell specialization and behavior. There also seems to be little doubt that if the level of oxygen available to the cell is elevated significantly in culture, both osteoclasis and chondroclasia ensue.[19, 32] In all probability, this pattern of specialization is attendant, in part, upon the activation of lysosomal enzymes within the cells.[35] Many other factors including parathyroid hormone, thyrocalcitonin, Vitamin A, antibodies, etc., introduced into the culture environment also affect profoundly the functional capacity of mesenchymal cells.[17]

In a recent paper entitled "Ultrastructural Aspects of Cartilage and Membrane Bone Differentiation from Common Germinal Cells," Hall and Shorey[21] trace the patterns of organelle development in osseous and cartilage cells in vitro. They conclude that:

> . . . the "throwing of the morphogenetic switch from osteogenesis to chondrogenesis" involves a change in the rate of collagen synthesis by the germinal cells, a high level of synthesis leading to the differentiation of bone and a low level of synthesis leading to the differentiation of adventitious cartilage.

One of the factors responsible for "throwing the morphogenetic switch" may be the nu-

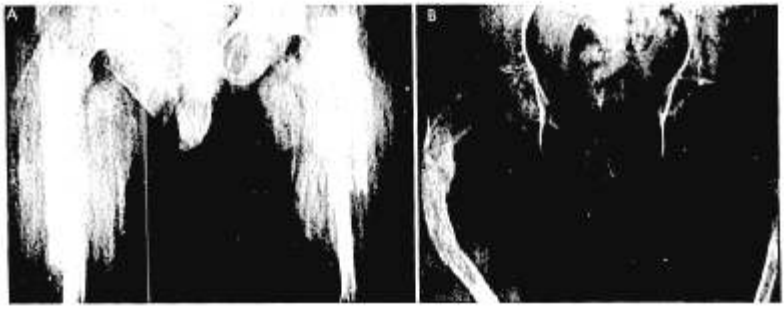

FIG. 5 A–B. A, Seven months (left) and 2 months (right) after realignment osteotomies and onlay grafts (freeze-dried, homogenous, cortical bone). Note irregular outlines of the graft on the left, which indicate centripetal revascularization and remodeling. B, Three years (left) and 2½ years (right) after grafting, none of the original onlay cortical grafts remains. They have been invaded and replaced (creeping substitution) with the abnormal osteogenesis imperfecta bone of the host. As a point of practical significance the patient achieved an ambulatory status for the first time, in braces, while the grafts were present.

tritional status of the cell. From tissue culture studies of chick cartilage it is evident that chondrocytes can be induced to form large quantities of collagen and, sometimes, to assume characteristics of osteocytes[3,7]. Furthermore, there is evidence from in vivo systems that fibrous tissue can be transformed into bone,[30] chondrocytes can be transformed directly to osteocytes,[14, 23] and the tissues in a pseudarthrosis (cartilage and fibrous tissue) can be transformed to bone without intercedent surgical excision or necrosis.[28]

This pattern of cell behavior should not be surprising if one considers that bone, cartilage and fibrous tissue have many biochemical similarities. Each contains collagen, mucopolysaccharides and minerals in slightly different proportions, types and combinations. At the ultrastructural level, bone is characterized by an intimate relationship between collagen fibrils and calcium hydroxyapatite crystals. Although this specific association is not found normally in other mammalian mesenchymal tissues, both collagen and mineral may co-exist in structures such as the calcified zone of the epiphyseal plate. It is conceivable, therefore, that major alterations in cell function probably are not necessary to produce a collagenous matrix which calcifies as opposed to one that doesn't. For example, it is known that variations in the type and amount of mucopolysaccharide present during reconstitution of collagen fibrils may account for physical differences in the resultant fibrils. Furthermore, it now appears probable that a mucopolysaccharide matrix exists in native collagenous fibrils and that the protein-polysaccharides may bear a structural relationship to collagen in the interfibrillar space. At the present time, there seems to be little question that the switch from osteogenesis to chondrogenesis in a population of germinal cells is the result of alterations in the metabolic pathways open to these mesenchymal cells.[20] These alterations are characterized by a suppression of collagen synthesis and a stimulation of acid mucopolysaccharide, mucoprotein, and glycogen production during chondrogenesis. Conversely, an increase in collagen synthesis and a suppresion of mucopolysaccharide production by these stem cells will result in osteogenesis.[20]

Obviously, the genomes of these stem, or germinal, cells permit synthesis of both mucopolysaccharides and collagen. The route of specialization is determined not only by nutritional factors but also by stimuli in the cell's environment. These stimuli may be chemical or physical.[3] Evidence, recently developed, suggest that certain chemical and physical stimuli may exert their influence, at least partly, through alterations in the electrical environment of the cell.[7, 12]

Studies in these and other laboratories during the past 60 years have established that bone functions as a transducer in converting mechanical to electrical energy. This finding has provided a better understanding of the mechanisms behind Wolff's Law,[6-8, 12] and has stimulated studies of electric effects on the behavior of connective tissue cells. Interestingly, in view of Hall's concepts of morphogenesis,[20, 21] exposure of cultures of fibroblasts to electrostatic fields, of an intensity well within physiologic range, will increase collagen synthesis greater than 100 per cent.[9] Dedifferentiation of cells, similar to that occurring in fracture healing, and collagen synthesis also have been induced by weak electric fields.[13] Further studies of this type may help to clarify differences in evolution of bone grafts placed in functional and nonfunctional environments, development of fibrous tissue in the face of net tensile forces, and the frequent appearance of cartilage in regions of shearing forces.

The pattern of specialization of mesenchymal cells in vitro has counterparts in many in vivo systems. For example, when an impermeable membrane, such as Silastic, is interposed between a region of osseous repair and the adjacent soft tissues, nutrition by extracellular fluids is diminished and cartilage frequently develops.[11, 25] Substitution of permeable Millipore for Silastic results in osteogenesis in these experimental systems, which are open to penetrating vessels. If osteogenic cells are completely isolated within Millipore diffusion chambers and implanted in the abdominal cavity, uniform bone formation occurs only as long as the space between the two facing chamber walls is sufficiently narrow and adequate nutrients are available. When the space exceeds a critical distance, islands or sheets of cartilage appear "sandwiched" between the two thin layers of bone which are located immediately adjacent to the sole source of nutritional extracellular fluids in the Millipore walls.[31]

Experimental bone transplants also demonstrate effects of mechanical factors on specialization of mesenchymal cells around and within the graft. Introduction of a length of fresh autologous fibula across the center of a rapidly growing epiphysis does not cause epiphyseodesis.[26] The two ends of the fibular segment in the metaphysis and bony epiphysis are fused to the host by callus, but the mid-portion is not fixed by callus to the cartilage of the epiphysis. Increasing tension, therefore, develops in the middle of the segment. Ordinarily, host cells invading pre-existing haversian systems in a bone graft are expected to specialize first as osteoclasts and secondly as osteoblasts in order to replace old bone with new. In this situation, however, osteoclasis in the middle of the graft was followed by fibroplasia as the mesenchymal cells, apparently responded to the net tensile forces in their immediate environment. Similar patterns of cellular responses to mechanical forces have been observed by Vigliani[30] in studies of spring-loaded rib grafts in animals. These investigations demonstrate, as do in vitro studies, that compression favors the specialization of osteoblasts and tension favors specialization of osteoclasts and fibroblasts.

It should be clear from the foregoing discussion that both nutritional and electromechanical factors are involved in osteogenesis during the early and late phases of bone grafting. Before leaving the subject of environmental effects on cell specialization, however, the role of noxious conditions or

toxic substances should be briefly considered. The pleuripotential capability of cells in a bone graft or its bed may be a disadvantage to the surgeon when this capability permits these cells to be diverted from forming bone by undesirable factors. For example, when the particle size of fresh autologous bone is reduced beyond a critical point, cells in the neighborhood are swept up in a granulomatous-foreign body response and do not form bone.[33] Addition of heteroantibody to a normally osteogenetic system results in a prompt cessation of osteogenesis and loss of cell "specialization".[18]

Nothing has been said here about the graft and its ability to induce osteogenesis. As adapted to bone by Urist and Adams,[38] the term "osteogenetic induction" can be summarized as follows: a mechanism of cellular differentiation or specialization in which there is an interaction between one tissue (the inductor) and another (the responding tissue) as a result of which the responding tissue takes a course of development it would not have followed if the interaction had not occurred. Much work has been done by Urist and his colleagues to define the nature of a bone induction principle (B.I.P.) in bone grafts and their derivatives.[39] Suffice it to say that several explanations of this phenomenon are possible. Surviving and *functioning* cells on the graft may be able to transfer B.I.P. to unspecialized cells in the pool by diffusion or by cell-to-cell contact. Although this mechanism depends upon graft viability, the two other main possibilities do not. First, it has been proposed that soluble (non-covalently bound) macromolecules released from the extracellular matrix of the graft during cellular invasion are capable of inducing osteogenesis.[39] Second, stress-generated electric potentials are thought to control osteogenesis.[6, 8, 12] Recently this concept has been adopted as a possible explanation for induction.[39] Under these circumstances, charge developed on the surface of the graft by functional mechanical or hemodynamic forces would induce cells in the pool to become osteogenic.[7] At the present time, however, the extent to which these mechanisms influence the outcome of a bone grafting procedure is not known.

Two differing patterns of bone graft evolution were demonstrated in Figures 1 and 2 at the beginning of this article. With the principles of cell–graft interaction outlined above, it should be possible now to understand the bases for the different graft behavior in these two cases. In the first patient, a dead, freeze-dried cortical homograft (allograft) was obtained from a bone bank and used to span the gap from femur to ischium. Less than 30 per cent of the graft was in contact with an osseous bed. Union between the host and both ends of the cortical strut, however, was achieved rapidly by callus formation. Following this, vascular penetration, together with its attendant waves of osteoclasis and osteogenesis, swept toward the center of the graft, successively weakening and strengthening it. The last portion to be reorganized, about 9 months after operation, was the center of the graft. As the full impact of mechanical force was focused in the reorganizing strut by weight-bearing, hypertrophy occurred. The second case, illustrated in Figure 2, also was a young boy. A piece of inadequately processed heterogenous (bovine) bone was placed in contact with an osseous bed throughout its length. It also was in a nonfunctional position. Normally, the heterograft (xenograft) would have been expected to be resorbed promptly, as did the fresh autograft placed just proximal to it. Quite probably, the foreign and denatured proteins in the bone were so noxious to the cells in the bed that an inflammatory response ensued. The bone remained unvascularized and, eventually, was walled-off. It therefore remained essentially unchanged and acted principally as a foreign body for a number of years. Under these circumstances it seems clear that the second patient got well "in spite"

of the cellular response to his biologically unacceptable graft, while the first patient got well because he received a biologically acceptable graft. The fate of the grafts in each of these cases could have been predicted with a high degree of accuracy before operation if the surgeons who were involved were armed with the principles of cell–graft interaction outlined above and the practical rules listed below.

PRACTICAL ASPECTS OF BONE GRAFTING

The first set of rules are designed to promote maximum survival of cells on the graft's surface. Here, a key consideration is the selection of a donor site with a large population of surface cells. Fresh autologous spongy bone from the iliac crest best meets this criterion. Once it has been removed, however, numerous precautions must be exercised in order to prevent death of surface cells. Although this last statement may seem superfluous, in the author's experience, many surgeons pay less attention to maintaining the viability of bone grafts than they do to skin grafts. During the interval between graft procurement and implantation, numerous hazards exist. Exposure to air for 30 minutes or more results in a significant decrease in cell viability.[1, 29] Physiologic saline solution is toxic after long-term exposure. Elevation of temperature above 42 C from direct exposure to operating room lights will kill cells. Many cold sterilizing agents (such as organic mercurials), bone wax, and a host of chemical agents (such as rust inhibitors used on scalpel blades) are lethal to cells in low concentrations. It also should be remembered that antibiotics (e.g., bacitracin and neomycin) are not only bactericidal, but "cellucidal" as well. Their use, therefore, in the presence of free, living, grafts must be undertaken with full awareness that they may diminish significantly the rate and bulk of callus formation.

Frequently, it is advantageous to "take" a graft early in the course of a procedure and hold it for use later in the operation. In view of the foregoing, how can a surgeon avoid hazards and assure a large population of viable cells? For intervals up to 4–6 hours, bone can be wrapped in a moistened, blood-soaked sponge and placed in a medicine cup or similar container, the top of which is covered by several saline-moistened sponges. Obviously, the container should not be exposed to heat. It is possible, when necessary, to save autologous bone for longer intervals and still maintain cell viability. This can be accomplished for periods up to several days by storage in 10 per cent human serum and 90 per cent balanced salt solution (Hank's, Earle's, or Ringer–Tyrode) at 3 C.

All measures taken before transplantation to maintain viability in a graft are useless unless sufficient amounts of nutrient are available in the recipient site. Several key steps can be taken to promote an effective diffusional nutrition in support of osteogenesis. First, prevent interposition of dead space, hematoma, or necrotic tissue between the graft(s) and its bed. Second, place cancellous surfaces of the graft next to the cancellous bone in the bed. Third, limit the thickness of cancellous grafts (not to include cortex or outer tables of the iliac crest) to a maximum of 5 mm, if a central zone of necrosis is to be circumvented.[36] The lateral dimensions (width and length) are not of practical importance in this instance. Fourth, do not permit the accumulative mass of grafts to become so thick and dense that diffusion from the underlying bony bed and overlying soft tissues is rendered ineffective. Fifth, survival of fresh autologous cancellous grafts can be improved when they are placed in a bed which already is actively producing new vessels and bone (e.g., beds prepared 2–3 weeks prior to grafting).[34]

Proper attention focused on these details will assure survival of a maximum number of cells on the surface of the graft. These cells, when added to the pool of cells in the bed, will increase the total number of *potential* osteoblasts. It is essential here to empha-

size the term "potential osteoblasts." As noted earlier, many factors, other than nutrition, can divert cells in the pool away from osteogenesis. The example, cited above, of excessively small particles of fresh bone inducing a foreign body or granuloma response is pertinent because it illustrates the need to consider cell function as well as survival. Generally, bone grafts should not be reduced to a size smaller than 1 cm^3.

In considering the long-term function of cells within an autograft, after physical union with the host is accomplished, mechanical factors play a dominant role. Grafts subjected to *net* compressive forces, in a physiologic range, probably will hypertrophy, while grafts subjected to no deforming force or a *net* tensile force may be replaced with nonosseous tissue. This mechanical influence on cell behavior dictates that close attention be paid not only to an analysis of forces acting in the recipient site, but also to the physical characteristics of the graft. Cancellous bone is more elastic (compliant) than is cortical bone. Narrow pieces of rigid cortical bone used as loaded struts (prop) in a bed of cancellous bone can focus excessive compressive, tensile and shearing forces on the supporting osseous tissues and the uniting callus with undesirable results. Not infrequently, skill in choosing grafts with optimal mechanical and microanatomical properties will determine the long-term cellular and clinical consequences of the procedure. As an example, consider a nonunion of the mid-radial shaft to be treated with an onlay graft. Here, since mechanical fixation is essential, a cortical graft from a site such as the tibia is proper. Osteogenesis can be buttressed by adding autologous cancellous chips or "matchsticks," if necessary. Use of fresh or preserved iliac crest bone as the sole source of internal fixation may result in failure because the small amount of osseous substance, present in the first place, will be further reduced as incorporation progresses. During reorganization, osteoclasis also will diminish the strength of the center portion of the cortical graft. This loss of substance occurs several months after grafting in a region already weakened by the nonunion and at a time when external support frequently is being discontinued. Clinical disaster may follow because excessive tensile and shearing forces applied to mechanically inadequate osseous tissue can result in fibrous tissue formation.

A comprehensive discussion of allografts is presented elsewhere.[10, 16] Fresh cancellous allografts behave initially in a manner similar to fresh cancellous autografts, until an immune rejection is triggered. This reaction can suppress or obliterate both graft and host osteogenic responses. *Fresh* homografts, either cancellous or cortical, therefore, are poor clinical choices. Since most preservation technics such as freezing or freeze-drying reduce graft antigenicity, host osteogenesis is of better quantity and duration when preserved homografts are substituted for fresh homografts. This fact is responsible, in large measure, for the circumscribed success of properly operated bone banks. Unlike cancellous grafts, the performance of preserved, *cortical,* homografts is very much similar to that of fresh autologous *cortical* grafts. Both are dead and dependent upon host cells for reorganization. Both can delay or stop their incorporation if not procured from proper sources or handled in a propitious manner. An excessive fat content or the presence of badly denatured matrix constituents can evoke inflammation, rather than osteogenesis. Boiling, autoclaving, merthiolate immersion, or storage at temperatures warmer than —28 C (the eutectic point of bone), all can produce a graft which places major obstacles in the way of the desired host osteogenic response.

SUMMARY

This article is a discussion of microenvironmental influences on cell behavior in the donor and recipient tissues including some basic principles of bone graft surgery.

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