Journal of Orthopaedic Research
9:641–650 Raven Press, Ltd., New York

Mesenchymal Stem Cells*

Arnold I. Caplan

The Skeletal Research Center, Department of Biology, Case Western Reserve University, Cleveland, Ohio, U.S.A.

Summary: Bone and cartilage formation in the embryo and repair and turnover in the adult involve the progeny of a small number of cells called mesenchymal stem cells. These cells divide, and their progeny become committed to a specific and distinctive phenotypic pathway, a lineage with discrete steps and, finally, end-stage cells involved with fabrication of a unique tissue type, e.g., cartilage or bone. Local cuing (extrinsic factors) and the genomic potential (intrinsic factors) interact at each lineage step to control the rate and characteristic phenotype of the cells in the emerging tissue. The study of these mesenchymal stem cells, whether isolated from embryos or adults, provides the basis for the emergence of a new therapeutic technology of self-cell repair. The isolation, mitotic expansion, and site-directed delivery of autologous stem cells can govern the rapid and specific repair of skeletal tissues. **Key Words:** Mesenchymal stem cells—Bone—Cartilage—Differentiation—Self-cell therapy—Skeletal tissue—Embryo—Adult.

THE CONCEPT

It is generally agreed that in an embryo a mesenchymal stem cell is a pluripotent progenitor cell which divides many times and whose progeny eventually gives rise to skeletal tissues: cartilage, bone, tendon, ligament, marrow stroma, connective tissue (Fig. 1). By definition, these stem cells are not governed by or limited to a fixed number of mitotic divisions. Their progeny are affected by a number of factors, however, as they become tracked into very specific developmental pathways in which both intrinsic and extrinsic factors combine to control the molecular and cellular pattern of expression that results in specific tissues that perform specific functions based on their molecular repertoire (9,11).

Received September 17, 1990; accepted January 17, 1991.

Address correspondence and reprint requests to Dr. Arnold I. Caplan at the Skeletal Research Center, Department of Biology, Case Western Reserve University, Cleveland, OH 44106, U.S.A.

* This publication was, in part, the basis for Dr. Caplan's receiving the Elizabeth Winston Lanier Award given by the American Academy of Orthopaedic Surgeons as part of their 1990 Kappa Delta Awards.

Indeed, the progression from stem cell to final end phenotype is marked by discrete stages with transit from one stage to the next dependent on local cuing from surrounding cells (paracrine regulation) as well as signals emitted by the cell itself and the reception of its own signaling (autocrine regulation) (10,57). The sum of these various intrinsic and extrinsic signals defines the developmental position of the cells. Although difficult to reconstruct on a cell culture dish, such "positional information" has been experimentally approached by studying embryonic cells in culture, cells that have the potential to differentiate into various phenotypes (7,9,11,15).

The concept of stem cells is now well established (21,60). Two systems serve as models for such a concept: First, *Caenorhabditus elegans* is a small worm whose entire developmental lineage map has been described (21); every cell found in the adult has been carefully tracked and its progenitor tree precisely established with every branch and subbranch delineated. Second, and to be emphasized, the heamtopoietic cell lineage has been described with its several diverging pathways (21,52). It is now clear that each separate pathway and, indeed,

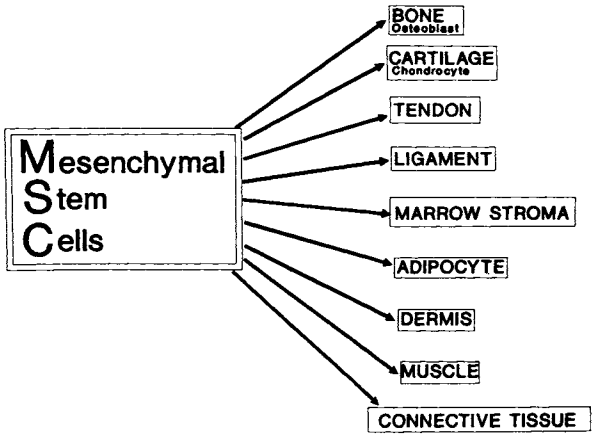

FIG. 1. Mesenchymal stem cell phenotypes. Mesenchymal stem cells are theoretically capable of differentiating through a series of separate and unique lineage transitions into a variety of end-stage phenotypes as shown.

progression through each separate stage within a discrete pathway is controlled by a balance of extrinsic and intrinsic macromolecules. Molecular biologists continue to isolate, clone, and express large amounts of these proteins, which allows use of cell culture systems to identify accurately the factor that controls progression to which stage and when (51,70). The challenge for skeletal biologists is to use the new information and new molecular tools to translate these advances into a better understanding of skeletal development, physiology, and repair.

EMBRYONIC MESENCHYMAL CELLS

The middle embryonic layer, the mesoderm, gives rise to all of the body's skeletal elements.* The term, mesenchyme, is derived from the Greek meaning "middle" (meso) "infusion" and refers to the ability of mesenchymatous cells to spread and migrate in early embryonic development between the ectodermal and endodermal layers. This characteristic migratory, space-filling ability is the key element of all wound repair in adult organisms involving mesenchymal cells in skin (dermis), bone (periosteum), or muscle (perimysium). Proteins that serve as chemoattractants, chemicals that specifically encourage this migratory activity to wound or developmental sites have been identified (24,32,59). The migratory activity of mesenchymal cells is complemented by their capacity to aggregate specifically to form unique developmental structures or, in adults, to form repair blastemas, which are then capable of responding to local cues and differentiating accordingly to achieve regenerative repair (10,11).

* For the sake of clarity, I address only issues related to cartilage or bone, although the same general experimental approach and logic can be used for other mesenchymal tissues.

Chick Limb Cells

More than 20 years ago, my collaborators and I attempted to define experimentally the conditions and cues necessary to control the differentiation of embryonic mesenchymal cells into cartilage and bone (5,7,17). Both in vivo and in vitro studies were used, but development of cell cultures and the general approach of using cell cultures has provided the experiential basis for approaching the study of mesenchymal stem cells from adults. The system we developed was the culturing of stage 24 (day 4.5) embryonic chick limb mesenchymal cells under conditions that promoted differentiation of cartilage (chondrocytes) (5,7,13,20) and bone (osteoblasts) (42,65).

Chondrocytes

Our first experimental effort with embryonic chick limb mesenchymal cells was to focus on chondrocyte development, which we learned was controlled by the initial plating density (5,17), oxygen levels (14), or, as recently shown by other investigators, a variety of physical and chemical factors (53,58,61). The key factor in the conversion of a mesenchymal cell to a chondrocyte is maintaining the progenitor cell in a round, unspread conformation. This can be accomplished simply by plating the cells initially under very compact, high-density conditions: 5×10^6 embryonic stage-24 limb mesenchymal cells per 35-mm dish (5,17). Even in a simple, defined medium consisting of insulin, transferrin, bovine serum albumin (BSA), and hydrocortisone in Eagle's minimum essential medium (MEM), the differentiation of chondrocytes and their further development can be documented as long as the cells are initially seeded at high density (18,30).

The high-density, limb cell-derived chondrocyte in culture makes two cartilage-specific molecules in abundance: type II collagen (68) and a large chondroitin sulfate, keratan sulfate proteoglycan (CSPG) (13,18,20). By detailed chemical and physical characterization of the CSPG synthesized on each day of culture, we showed that the glycosaminoglycan chains are biosynthesized slightly differently with

time (Fig. 2). Peptide maps show that the newly synthesized core protein (26) is identical on each day of culture, whereas the chondroitin sulfate chains are synthesized progressively shorter (30,000 D on day 2 to 15,000 D on day 20) and the keratan sulfate chains are synthesized progressively larger (0 to 10,000 D) (13,20). This biosynthetic progression is exactly what has subsequently been shown to occur in the cartilages of embryonic, adult, and aging human (50) and bovine specimens (62).

That embryonic chondrocytes have an aging-dependent program of changing biosynthesis is further documented when cultured embryonic chick chondrocytes are transplanted in a fibrin-based delivery vehicle into defects at the articular surface of adult chickens (29). Such chondrocytes produce what appears to be appropriate cartilaginous matrix and have been followed >18 months. The resulting repair cartilage appears to integrate perfectly into the defect and to provide the animal with a healthy, normal articular surface. These experiments and others clearly establish the concept of repairing cartilage with embryonic or appropriate reparative cells.

Osteoblasts

Our initial success in studying emergence of chondrocytes and formation of cartilaginous tissue from cultures of limb mesenchymal cells encouraged us to study differentiation of osteoblasts and formation of bone as well. Our initial logic was that high-density conditions caused cartilage formation and that cartilage was the progenitor tissue of bone. (Some investigators have reported that cartilage provides the scaffold for bone formation.) After 2 years of frustrating experimentation, we realized that when infrequent bone and osteoblasts could be identified, the bone had formed at a distance from cartilage and never on or in the cartilage (42). By carefully decreasing the initial cell density of limb mesenchymal cells to just below the density at which some mineralized cartilage could form (2×10^6 cells/35-mm dish), we observed numerous deposits of bone and abundant osteoblasts which, again, were clearly at some distance from cartilage (6,42,44). In addition, these osteoblasts exhibited the classic response to parathyroid hormone (PTH) of elevated cyclic AMP levels (71,72) and possessed a bone-specific alkaline phosphatase (43). These studies clearly indicated that embryonic chick limb mesenchymal cells were capable of differentiating into osteoblasts and that the culture conditions supporting optimum osteoblast emergence were different from the conditions optimum for chondrogenesis.

Mouse and Human Limb Cells

With regard to cartilage and bone, the properties of mouse and human limb mesenchymal cells in culture appear to be quite similar, if not identical (25,46). Likewise, cartilage and bone development in vivo are also quite comparable, with the major exception that embryonic cartilage of chick does not calcify whereas that of mammals always calcifies (16). The comparable developmental properties of aves, rodents, and humans encourages us to continue experimentation with animal cells as an approximation of better understanding of the properties of human cells and tissues.

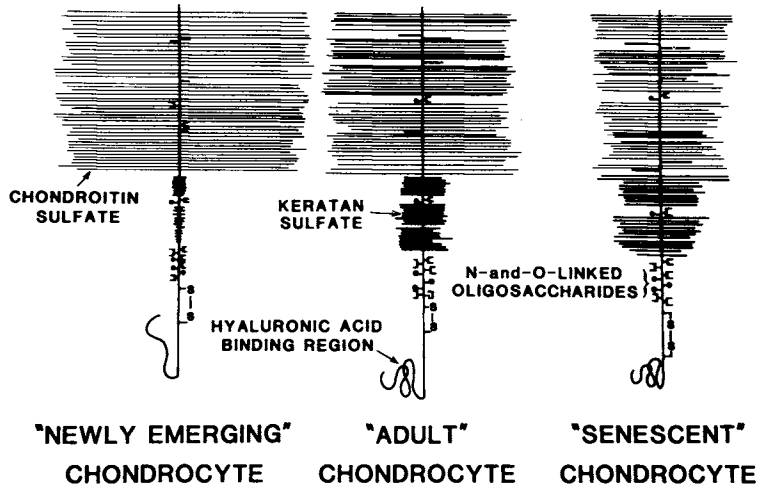

FIG. 2. Proteoglycans synthesized by newly differentiated, mature, and senescent chondrocytes. With increasing age, chondrocytes synthesize proteoglycans that have smaller chondroitin sulfate chains and larger keratan sulfate chains (7,8,12,13,20).

LINEAGE OF MESENCHYMAL CELLS

Cartilage

The important inference from the above discussion is that chondrocytes have a programmed (i.e., genetically dictated) sequence of changes in their end-stage expression (8,12). The differences in glycosaminoglycan chain lengths or chemistry are stable to cell culturing or metabolic perturbation. The control of these events is not known, but all experiments designed to slow this sequence of biosynthetic alterations or reverse them have failed. The inference is that a genomic mechanism somehow "tells time" and that this clock is hard-wired and unidirectional (8,12).

Such biosynthetic changes in articular cartilage are different from the lineage changes observed in adult growth plate or embryonic limb cartilage. A discrete set of expressional stages or lineage states, comprising dividing, maturing, and hypertrophic chondrocytes, is apparent in embryonic limb tissue, cell culture (13,58,61), and in the growth plate (19,28). Eventually, the hypertrophic cartilage in vivo is eroded by vascular, marrow, and phagocytic cells and replaced by bone. Each chondrocytic lineage state is uniquely different from its predecessor, as shown in Fig. 3. For example, hypertrophic chondrocytes synthesize a unique small collagen, type X, and a unique proteoglycan (54,55); neither of these molecules is synthesized by mature chondrocytes. In this particular circumstance, several factors are proposed to contribute to conversion of mature chondrocytes to hypertrophic chondrocytes (35); reversal of this process has not been reported.

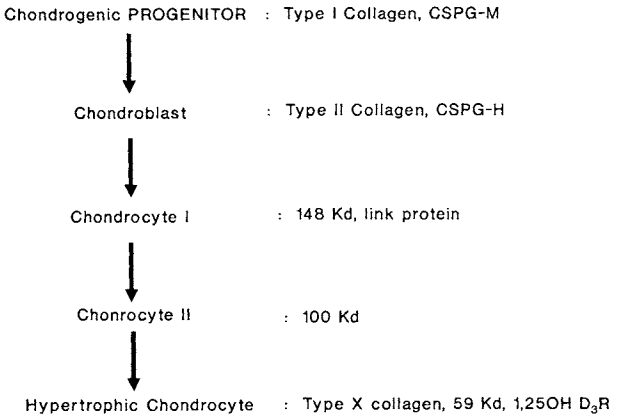

FIG. 3. Chondrogenic lineage. Based on the experiments of Solursh et al. (58,61) a hypothetical lineage map can be constructed to consist of at least five separate stages based on the changing biosynthesis of proteins (named or by molecular weight, K_d) or chondroitin sulfate proteoglycan (CSPG). The receptor for 1,25-dihydroxy Vitamin D_3 is represented as $1,25OHD_3R$.

Bone

We recently reviewed the major aspects of embryonic bone development. Figure 4 shows several important elements or rules governing this complex process (10,11,16). First, a discrete positioning of progenitor cells, stacked cells, existed in proximity to the developing bone (47). The stacked cells give rise to osteoblats in a discrete series of lineage steps (described below). The end stage or secretory osteoblast is positioned by its proximity to vasculature, with the "back" of the osteoblast to the capillary and osteoid deposited from the "front" of this highly oriented secretory cell (47,48). The vasculature is the orientor of osteogenesis and the osteoblast is the formative element. Cartilage is not replaced by bone, but is instead the target for vascular (marrow) replacement (48); in the early limb, the cartilage model exactly defines the eventual marrow cavity.

That a discrete series of individual lineage stages exists between the progenitor cells in the stacked cell layer and the secretory osteoblasts is now clear, as shown in Fig. 5. We recently isolated four monoclonal antibodies, SB1, 2, 3, and 5, which have helped provide evidence for an osteoblast lineage (3,4). Progenitor cells in the stacked cell layer and osteocytes do not interact with SB1, 2, or 3. Newly differentiated osteogenic cells react with SB1, but not with SB2 or 3, whereas fully secretory osteoblasts react with SB1, 2, and 3. A subpopulation of osteogenic cells reacts with SB2, but not SB3. Osteocytes react with OB7.3 of Nijweide and Mulder (38) or with our SB5, but not with SB1, 2, or 3. The lineage tree in Fig. 3 is based on these observations and not only establishes the existence of an osteoblastic lineage but suggests that osteocytes are derived directly from osteoblasts with SB1, 2, and 3 antigens that are suppressed as SB5 and OB7.3 are turned on. Experiments are now in progress to use these monoclonal antibodies to isolate representatives of each lineage stage so that studies can be conducted to identify the agents that promote the progression from one lineage stage to the next. Central to the thesis presented below is the existence of osteoprogenitor cells in the stacked cell layer, the future periosteum.

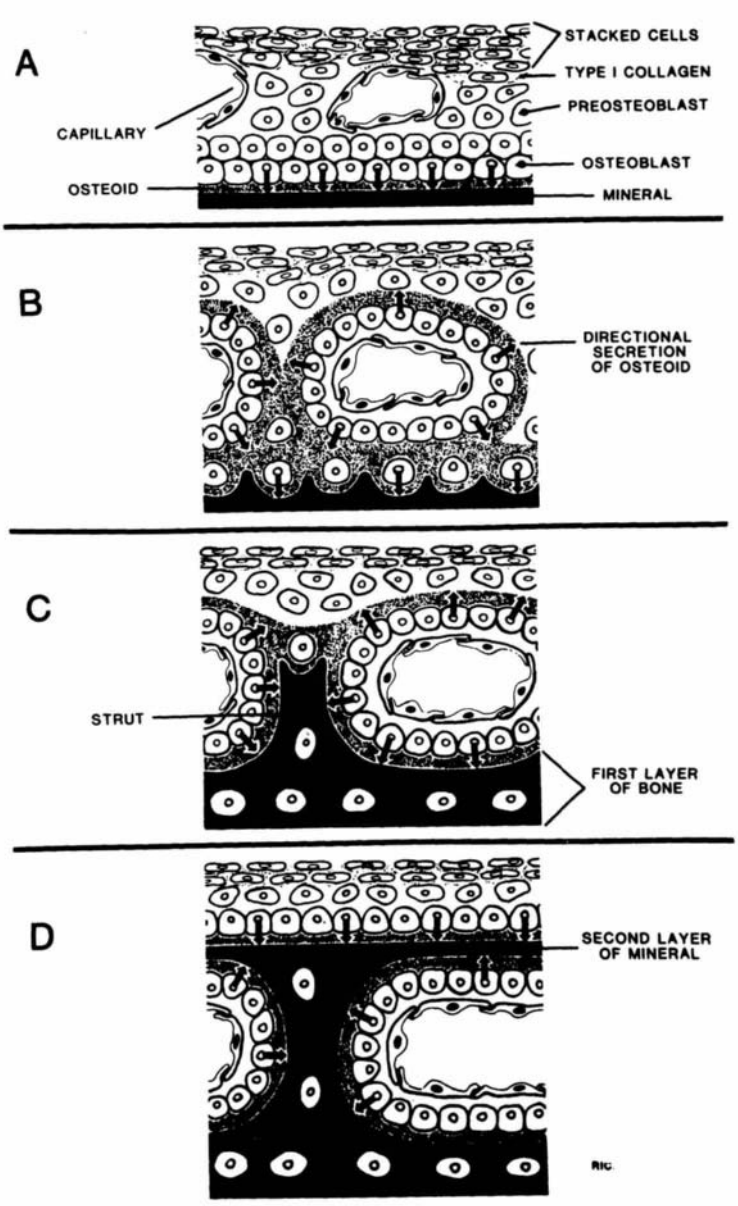

FIG. 4. Sequence of progressive in vivo bone development. Progressive repositioning of the vasculature from outside the stacked cell layer to a position in close approximation to the first layer of secretory osteoblasts responsible for formation of the first bony collar of the chick tibia (11,47,48). The osteoblast is oriented with its back toward the invading capillary and secretion of osteoid toward the cartilage core from the osteoblast's face. In this model, osteoblasts secrete osteoid in a direction away from vasculature **(B),** causing formation of a strut **(C)** and eventually forming the second layer of bone **(D).** These observations show that an intimate relationship exists between vasculature and newly forming bone.

BIOACTIVE FACTORS IN BONE

From the earliest days of modern humans, bone has been recognized to have the powerful capacity to repair discontinuities (22). A variety of bioactive factors combine in a complex multicellular, multistep response in which reparative cells are specifically attracted to the repair site. These cells then aggregate, multiply, bridge the bone gap, and differentiate into chondrocytes or osteoblasts as controlled by the proximity to vasculature. Recently, an intensive research activity to identify and characterize these various bioactive factors was largely successful (56,66,67,69). Our laboratory has described the purification of a protein factor, chondrogenic stimulating activity (CSA), which converts embryonic limb mesenchymal cells to chondrocytes (63,64). We are also attempting to purify a bone-derived chemoattractant for mesenchymal cells by using the now standard modified Boyden chamber (31,33).

Relevant to the thesis developed below, the identity and manipulation of the cells responding to bone-derived bioactive factors is directly related to successful bone repair. Such responding cells are present in the adult periosteum (36), dermis (49),

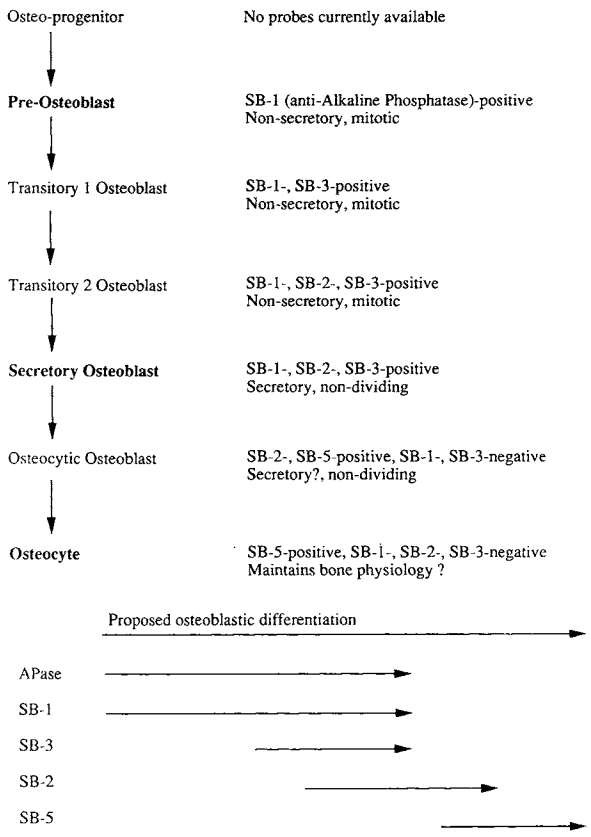

FIG. 5. Osteogenic cell lineage. Based on recent experimentation in which monoclonal antibodies were generated to cell surface antigens of osteogenic cells (3,4), a tentative lineage diagram reflects acquisition or loss of specific antigenic determinants. The characteristics of SB1, 2, and 3 were described previously (3); SB-5 (4) has been characterized and is similar to OB7.3 of Nijweide and Mulder (38). The individual lineage states are not weighted in terms of their prevalence or dwell-time; e.g., "transitory osteoblast 1" occurs rarely and cannot be recognized easily except at specific times and locations, whereas the "secretory osteoblast" is easily recognized and plentiful.

bone marrow (1,40,41,45), and connective tissue associated with muscle (34,37). One or all of these repositories are capable of forming bone when appropriately delivered bioactive factors are presented.

Alternately, when the responsive cells, stem cells, are placed in suitable delivery vehicles that can retain these cells while encouraging vascular invasion, bone can be observed to form. Recently, we used calcium phosphate porous ceramics in composite with marrow to encourage bone formation at both heterotopic and orthotopic sites (40,41). Whole disaggregated marrow cells in suspension are loaded into porous ceramic and transplanted to subcutaneous, intramuscular, or bone defect sites in vivo. In 1–2 months, the few mesenchymal stem cells in the marrow have replicated massively and differentiated into osteoblasts. In the dead-end pores of the ceramic, which are devoid of vasculature, these stem cells differentiate into chondrocytes and form cartilage.

MESENCHYMAL STEM CELLS

From the above discussion several key facts are evident. First, embryonic mesenchymal stem cells in the limb which give rise to cartilage and bone in vivo can be manipulated in vitro. Second, these cells have a lineage progression of separate, individual steps, whether it be the chondrogenic or osteogenic pathway. Third, local cuing, sometimes involving highly potent protein factors, is responsible for providing positional information and causing lineage progression. Cell culture conditions have been refined to the extent that not only can these progressive events be studied in detail, but manipulation of the cells is also possible to provide control of tissue size and function.

Fourth, although chondrocytes and osteoblasts are derived from a common mesenchymal cell, the conditions for their initial differentiation and progression through the individual steps of their lineages are uniquely different. For example, osteogenesis is dependent on proximity to vasculature whereas chondrogenesis requires the complete absence of vasculature (7,10,11,16); osteogenesis is optimum at an initial cell culture seeding density in 35-mm dishes of 2×10^6 embryonic limb mesenchymal cells, whereas chondrogenesis is optimum at 5×10^6 cells (5,17,42).

Fifth, bone forms from mesenchymal stem cells in a cartilage-independent manner with vasculature providing a determinative discriminator between these two tissues; embryonic cartilage is not replaced by bone, but rather by vasculature and marrow (10,11,16). Sixth, we can demonstrate that three tissue sites are the repositories of mesenchymal stem cells: marrow (1,40,41,45), periosteum (36), and muscle connective tissue (34,37).

MARROW

Figure 6 outlines an assay to demonstrate that marrow contains mesenchymal stem cells capable of differentiation into cartilage and bone. Whole marrow is disrupted into single cells by passing it through needles of successively smaller sizes; the

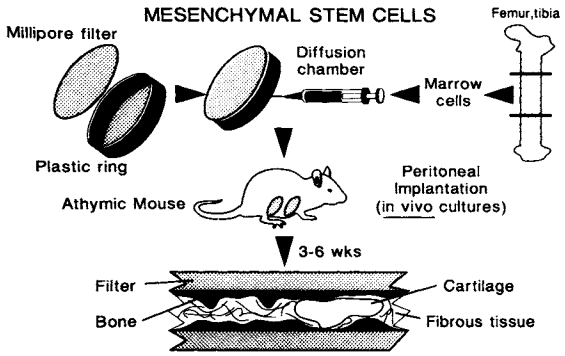

FIG. 6. Diffusion chamber assay in nude mice. Cell samples from marrow or other sources can be loaded into chambers composed of two Millipore filters glued to the edges of a plastic ring. These chambers are then implanted in the peritoneal cavity of athymic (nude) mice as a highly vascular in vivo incubation site. The filters prevent host cells from entering the chambers but permit rapid diffusion of nutrients and other factors into or out of the chamber. Histologic identification of two distinctive phenotypes, cartilage and bone, indicates that mesenchymal stem cells were present in the initial inoculum (1,2,45).

cells are counted, and 1–10 $\times$ 10^6 cells are placed in a small diffusion chamber (1,2,45). This chamber is of simple construction consisting of a small plastic ring onto which two Millipore filters have been glued. The filters allow body fluids (salts, nutrients, proteins, large protein complexes) to pass in and out of the chamber, but cells inside are not mixed with host cells, and tissues such as the vasculature are completely excluded. These chambers are implanted into the peritoneal cavity of an athymic (nude) mouse as an in vivo incubator, and they quickly become surrounded by host vasculature. Detailed studies have shown that the hematopoietic cells are eliminated, whereas mesenchymal cells vigorously divide and differentiate into cartilage in the middle of the chamber and bone at the filter interfaces closest to the enveloping vasculature (1,2,45). The presence of both cartilage and bone in the diffusion chamber has been compared to the presence of predominantly bone inside the highly vascularized pore regions of porous calcium phosphate ceramics loaded with marrow cells and implanted at heterotopic or orthotopic sites described above (40,41).

As a refinement of these experiments, we have been able to purify marrow mesenchymal cells by their differential adhesion to culture dishes and have successfully cultured cells through many passages (23). These cultured marrow mesenchymal cells from rat or chicken retain their capacity to differentiate into osteoblasts in ceramics through such subculturing. Of importance is the demonstrated success of isolating marrow mesenchymal cells and mitotically expanding these cells with retention of their full developmental potency to differentiate into osteoblasts or chondrocytes.

Periosteum

Another repository for mesenchymal stem cells is the periosteum, a complex layer of cells that composes the outermost layer of long bone; we have termed the periosteum the stacked cell layer in developing embryos (1,16,47,48). This layer clearly responds to injury by rapidly expanding and forming woven bone; it also has cells capable of differentiating into chondrocytes when the periosteum is transplanted into an articular cartilage defect (39). In experimentation paralleling that described above for marrow mesenchymal cells,we have been successful in culturing and passaging periosteal cells (36). In porous ceramics implanted in nude mice, these cultured periosteal cells differentiate into osteoblasts (36). When the same cell preparation is injected into a subcutaneous site in a nude mouse, the cultured periosteal cells differentiate into both bone and cartilage (36). The important point is that culture-expanded periosteal cells retain their full developmental potency and can be manipulated to form two very complex and different tissues, bone or cartilage.

THE FUTURE: (SELF-CELL THERAPY)

Several important conceptual and technical advances have converged to allow us to consider the possibility of using a patient's own mesenchymal stem cells as starting material for tissue repair protocols. Mesenchymal stem cells must exist to maintain the living organisms, just as hematopoietic stem cells must exist to support both red and white blood cell turnover. Developmental biology has taught us that differentiated cells arise in a sequence of definitive cellular and molecular transitions, a lineage, from stem cell to end phenotype. Bone, for example, turns over; new osteoblasts arise, have a defined half-life, make new bone, and then die, to be replaced by other newly differentiating end-stage osteoblasts. Such osteoblasts must arise from stem cells; thus, a living organism must have repositories of stem cells.

Therefore, we might be able to isolate such human mesenchymal stem cells and place them in cell

culture, where we could mitotically expand their numbers. Eventually, if we had enough of these cells, we could reintroduce them into the original donor in a manner that guaranteed that they would massively differentiate into a specific tissue, such as cartilage or bone, at a transplantation or repair site. Immunorejection would not be a problem because the donor and host would be one and the same.

The first experimental step to test this idea is to determine if the animal-based technology described above can be modified to be used with human material. The first attempts at this have been highly encouraging. Recently, human marrow was introduced into diffusion chambers which were placed in nude mice; both cartilage and bone were eventually observed in the chamber (2). We recently cultured human marrow and isolated mesenchymal cells that were passaged, introduced into porous ceramics, and implanted subcutaneously in nude mice. In the pore regions of these highly vascularized composites, bone clearly formed in every sample of culture-expanded, marrow-derived mesenchymal cells tested (27). These preliminary experiments provide hope that the animal-based technology developed for mesenchymal cells from marrow or periosteum will be translatable to humans.

The concept of ex vivo manipulation of cells and their reimplantation into a donor is the basis for proposing self-cell therapy as a future possibility. Massive bone regeneration to fill gaps from tumor excision, regeneration of damaged articular cartilage, and maintenance of bone formation in the elderly at risk for osteoporosis are clinical protocols that require large numbers of the appropriate reparative skeletal cells. The patient's own mesenchymal stem cells may prove to be the basis of a new, cell-based treatment plan requiring the merging of molecular biology to produce specific bioactive factors, cell biology to develop ex vivo manipulation regimens, and surgeons able to implant cells capable of repairing skeletal defects by the regeneration process.

Acknowledgment: I thank the members of my laboratory, both past and present, for providing the fabric and labor of the cloth of our scientific pursuits. The resultant material of many colors provides both the backdrop and carpet for our scientific accomplishments and progress. My thanks are not enough to repay their kindnesses, contributions, and stimulation. This work was supported by grants from the NIH.

REFERENCES

1. Bab I, Howlett CR, Ashton BA, Owen ME: Ultrastructure of bone and cartilage formed in vitro in diffusion chambers. *Clin Orthop* 187:243–254, 1984
2. Bab I, Passi-Even L, Gazit D, Sekeles E, Ashton BA, Peylan-Ramu N, Ziv I, Ulmansky M: Osteogenesis in in vivo diffusion chamber cultures of human marrow cells. *Bone Mineral* 4:373–386, 1988
3. Bruder SP, Caplan AI: First bone formation and the dissection of an osteogenic lineage in the embryonic chick tibia is revealed by monoclonal antibodies against osteoblasts. *Bone* 10:359–375, 1989
4. Bruder SP, Caplan AI: Terminal differentiation of osteogenic cells in the embryonic chick tibia is revealed by a monoclonal antibody against osteocytes. *Bone* 11:189–198, 1990
5. Caplan AI: Effects of the nicotinamide-sensitive teratogen 3-acetyl-pyridine on chick limb cells in culture. *Exp Cell Res* 62:341–355, 1970
6. Caplan AI: Muscle, cartilage and bone development and differentiation from chick limb mesenchymal cells. In: *Vertebrate Limb and Somite Morphogenesis,* ed by DA Ede, JR Hinchliffe, and M Balls, Cambridge, England, Cambridge University Press, 1977, pp 199–213
7. Caplan AI: The molecular control of muscle and cartilage development. In: *39th Annual Symposium of the Society for Developmental Biology,* ed by S Subtelney, U Abbott, New York, Alan R. Liss, 1981, pp 37–68
8. Caplan AI: Cartilage. *Scientific American* 251:84–94, 1984
9. Caplan AI: The extracellular matrix is instructive. *BioEssays* 5:129–132, 1986
10. Caplan AI: Bone development and repair. *BioEssays* 6:171–175, 1987
11. Caplan AI: Bone development. In: Wiley J, ed. *Cell and Molecular Biology of Vertebrate Hard Tissues.* Chichester, CIBA Foundation Symposium 136. 1988:3–21.
12. Caplan AI, Fiszman MY, Eppenberger HM: Molecular and cell isoforms during development. *Science* 221:921–927, 1983
13. Caplan AI, Hascall VC: Structure and developmental changes in proteoglycans. In: *Dilatation of the Uterine Cervix,* ed by F Naftolin, and PG Stubblefield, New York, Raven Press, 1980, pp 79–98
14. Caplan AI, Koutroupas S: The control of muscle and cartilage development in the chick limb: The role of differential vascularization. *J Embryol Exp Morphol* 29:571–583, 1973
15. Caplan AI, Ordahl CP: Irreversible gene repression model for control of development. *Science* 201:120–130, 1978
16. Caplan AI, Pechak DG: The cellular and molecular embryology of bone formation. In: *Bone and Mineral Research,* vol. 5, ed by WA Peck, Elsevier, New York, 1987, pp. 117–184
17. Caplan AI, Zwilling E, Kaplan NO: 3-Acetylpyridine: effects in vitro related to teratogenic activity in chicken embryos. *Science* 160:1009–1010, 1968
18. Carrino DA, Kujawa MJ, Lennon DP, Caplan AI: Altered proteoglycans synthesized by chick limb bud chondrocytes cultured in serum-free defined medium. *Exp Cell Res* 183:62–71, 1989
19. Cruz-Orive L-M, Hunziker EB: Steriology for anisotropic cells: Application to growth cartilage. *J Microsc* 143:47–80, 1986
20. DeLuca S, Heinegard D, Hascall VC, Kimura JH, Caplan AI: Chemical and physical changes in proteoglycans during development of chick limb bud chondrocytes grown in vitro. *J Biol Chem* 252:6600–6608, 1977

21. Dexter TM, Spooncer E: Growth and differentiation in the hematopoietic system. *Annu Rev Cell Biol* 3:423–441, 1987
22. Goldberg K, and editors of U.S. News: The human body: The skeletal-fantastic framework. *U.S. News* 1982
23. Goshima J, Goldberg VM, Caplan AI: The osteogenic potential of culture-expanded rat marrow mesenchymal cells as assayed in vivo in calcium phosphate ceramic blocks. *Clin Orthop Related Res* 262:298–311, 1991
24. Grotendorst GR, Martin GR: Cell movements in wound healing and fibrosis. *Rheumatology* 10:385–403, 1986
25. Hauschka SD: Clonal analysis of vertebrate myogenesis, III. Developmental changes in the muscle colony-forming cells of the human fetal limb. *Dev Biol* 37:345–368, 1974
26. Haynesworth SE, Carrino DA, Caplan AI: Comparison of the cartilage proteoglycan core protein synthesized by chondrocytes of different ages. *Connect Tissue Res* 25:311–320, 1991
27. Haynesworth SE, Goshima J, Goldberg VM, Caplan AI: Isolation and expansion of cells with osteogenic potential from human marrow. *Bone* (submitted for publication)
28. Hunziker EB: Growth plate structure and function. *Pathol Immunopathol Res* 7:9–13, 1988
29. Itay S, Abramovici A, Nevo Z: Use of cultured embryonal chick epiphyseal chondrocytes as grafts for defects in chick articular cartilage. *Clin Orthop* 220:284–303, 1987
30. Kujawa MJ, Lennon DP, Caplan AI: Growth and differentiation of stage 24 limb mesenchymal cells in a serum-free, chemically defined medium. *Exp Cell Res* 183:45–61, 1989
31. Lucas PA, Caplan AI: Chemotactic response of embryonic limb bud mesenchymal cells and muscle-derived fibroblasts to transforming growth factor beta. *Connect Tissue Res* 18:1–7, 1988
32. Lucas PA, Price PA, Caplan AI: Chemotactic response of mesenchymal cells, fibroblasts and osteoblast-like cells to bone GLA protein. *Bone* 9:319–323, 1988
33. Lucas PA, Syftestad GT, Caplan AI: Partial isolation and characterization of a chemotactic factor from adult bone for mesenchymal cells. *Bone* 7:365–371, 1986
34. Lucas PA, Syftestad GT, Caplan AI: A water-soluble fraction from adult bone stimulates the differentiation of cartilage in explants of embryonic muscle. *Differentiation* 37:47–52, 1988
35. Manduca P, Castagnola P, Cancedda R: Dimethyl sulfoxide interferes with in vitro differentiation of chick embryo endochondral chondrocytes. *Dev Biol* 125:234–236, 1988
36. Nakahara H, Bruder SP, Goldberg VM, Caplan AI: In vivo osteochondrogenic potential of cultured cells derived from the periosteum. *Clin Orthop Rel Res* 259:223–232, 1990
37. Nathanson MA, Hay ED: Analysis of cartilage differentiation from skeletal muscle grown in bone matrix I. Ultrastructural aspects. *Dev Biol* 78:301–331, 1980
38. Nijweide P, Mulder R: Identification of osteocytes in osteoblast-like cell cultures using a monoclonal antibody specifically directed against osteocytes. *Histochemistry* 84:342–347, 1986
39. O'Driscoll SW, Salter RB: The induction of neochondrogenesis in free intra-articular periosteal autografts under the influence of continuous passive motion. *J Bone Joint Surg [Am]* 66:1248, 1984
40. Ohgushi H, Goldberg VM, Caplan AI: Heterotopic osteogenesis in porous ceramics induced by marrow cells. *J Orthop Res* 7:568–578, 1989
41. Ohgushi H, Goldberg VM, Caplan AI: Repair of segmental long bone defect by composite graft of marrow cells and porous calcium phosphate ceramic. *Acta Orthop Scand* 60:334–339, 1989
42. Osdoby P, Caplan AI: Osteogenesis in cultures of limb mesenchymal cells. *Dev Biol* 73:84–102, 1979
43. Osdoby P, Caplan AI: Characterization of bone-specific alkaline phosphatase in cultures of chick limb mesenchymal cells. *Dev Biol* 86:136–146, 1981
44. Osdoby P, Caplan AI: First bone formation in embryonic chick limbs. *Dev Biol* 86:147–156, 1981
45. Owen M: Lineage of osteogenic cells and their relationship to the stromal system. In: *Bone and Mineral Research,* Vol. 3, ed by WA Peck, New York, Elsevier, 1985, pp 1–25
46. Owens EM, Solursh M: In vitro histogenic capacities of limb mesenchyme from various stage mouse embryos. *Dev Biol* 88:297–311, 1981
47. Pechak DG, Kujawa MJ, Caplan AI: Morphological and histochemical events during first bone remodeling in embryonic chick limbs. *Bone* 7:441–458, 1986
48. Pechak DG, Kujawa MJ, Caplan AI: Morphology of bone development and bone remodeling in embryonic chick limbs. *Bone* 7:459–472, 1986
49. Reddi AH: Cell biology and biochemistry of endochondral bone development. *Coll Rel Res* 1:209–226, 1981
50. Roughley PJ, White RJ: Age-related changes in the structure of the proteoglycan subunits from human articular cartilage. *J Biol Chem* 255:217–224, 1980
51. Sachs L: Hematopoietic growth and differentiation factors after the reversal of malignancy. In: *Tumor Cell Differentiation,* ed by J Aarbakke, PK Chiang, and HP Koeffler, Clifton, NJ, Humana Press, 1987, pp 3–27
52. Sachs L: The molecular control of blood cell development. *Science* 238:1374–1379, 1987
53. San Antonio JD, Tuan RS: Chondrogenesis of limb bud mesenchyme in vitro: stimulation by cations. *Dev Biol* 115:313–324, 1986
54. Schmid TM, Conrad HE: Metabolism of low molecular weight collagen by chondrocytes obtained from histologically distinct zones of the chick embryo tibiotarsus. *J Biol Chem* 257:12451–12457, 1982
55. Schmid TM, Linsenmayer TF: Developmental acquisition of type X collagen in the embryonic chick tibiotarus. *Dev Biol* 107:373–381, 1985
56. Seyedin SM, Thomas TC, Thompson AY, Rosen DM, Piez KA: Purification and characterization of two cartilage-inducing factors from bovine demineralized bone. *Proc Natl Acad Sci USA* 82:2267–2271, 1985
57. Slavkin HC: Molecular biology of dental development: a review. In: *The Biological Mechanism of Tooth Eruption and Root Resorption,* ed by Z Davidovitch, EbscoMed, Birmingham, AL, 1988, pp 107–116
58. Solursh M, Reiter RL, Ahrens PB, Vertel BM: Stage- and position-related changes in chondrogenic response of chick embryonic wing mesenchyme to treatment with dibutyryl cyclic AMP. *Dev Biol* 83:9–19, 1981
59. Somerman M, Hewitt AT, Varner HH, Schiffman E, Termine J, Reddi AH: Identification of a bone matrix-derived chemotactic factor. *Calcif Tissue Int* 35:481–485, 1983
60. Sulston J, Schierenberg E, White J, Thomson J: The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Dev Biol* 100:64–119, 1983
61. Swalla BJ, Solursh M: Inhibition of limb chondrogenesis by fibronectin. *Differentiation* 26:42–48, 1984
62. Sweet MBE, Thomar EJ-MA, Marsh J: Age-related changes in proteoglycan structure. *Arch Biochem Biophys* 198:439–448, 1979
63. Syftestad GT, Caplan AI: A fraction from extracts from demineralized bone stimulates the conversion of mesenchymal cells into chondrocytes. *Dev Biol* 104:348–356, 1984

64. Syftestad GT, Lucas PA, Caplan AI: The in vitro chondrogenic response of limb bud mesenchyme to a water soluble fraction prepared from demineralized bone matrix. *Differentiation* 29:230–237, 1985
65. Syftestad GT, Weitzhandler M, Caplan AI: Isolation and characterization of osteogenic cells derived from first bone of the embryonic tibia. *Dev Biol* 110:275–283, 1985
66. Takaoka K, Ono K, Ametani K, Kishemoto R, Nakata Y: Solubilization and concentration of bone-inducing substance from a murine osteosarcoma. *Clin Orthop* 148:274–280, 1980
67. Urist MR, Delange RJ, Finerman GAM: Bone cell differentiation and growth factors. *Science* 220:680–686, 1983
68. von der Mark K, Osdoby P, Caplan AI: Effect of 4-methyl umbellifer-yl-β-D-xyloside on collagen synthesis in chick limb bud mesenchymal cell cultures. *Dev Biol* 90:24–30, 1982
69. Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Kewick RM, Wang EA: Novel regulators of bone formation: Molecular clones and activities. *Science* 242:1528–1534, 1988
70. Zipori D, Lee F: Introduction of interleukin-3 gene into stromal cells from the bone marrow alters hematopoietic differentiation but does not modify stem cell renewal. *Blood* 71:586–596, 1988
71. Zull JE, Krug S, Abel D, Caplan AI: Development of parathyroid hormone and calcitonin-activated adenylate cyclases in the embryonic limb and in cultured cells from embryonic chick limb. *Proc Natl Acad Sci USA* 75:3871–3875, 1978
72. Zull JE, Youngman K, Caplan AI: The development of hormonal responses of cultured embryonic chick limb mesenchymal cells. *Dev Biol* 86:61–68, 1981