An Overview of Vertebrate Mineralization with Emphasis on Collagen-Mineral Interaction
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ABSTRACT
The nucleation, growth, and development of mineral crystals through their interaction principally with collagen in normal bone and calcifying tendon have been elaborated by applying a number of different techniques for analysis of the inorganic and organic constituents of these tissues. The methods have included conventional and high voltage electron microscopy, electron diffraction, microscopic tomography and 3D image reconstruction, and atomic force microscopy. This summary presents results of these studies that have now characterized the size, shape, and aspects of the chemical nature of the crystals as well as their orientation, alignment, location, and distribution with respect to collagen. These data have provided the means for understanding more completely the formation and strength of the collagen-mineral composite present in most vertebrate calcifying tissues and, from that information, a basis for the adaptation of such tissues under mechanical constraints. In the context of the latter point, other data are given showing effects on collagen in bone cell cultures subjected to the unloading parameters of spaceflight. Implications of these results may be particularly relevant to explaining loss of bone by humans and other vertebrate animals during missions in space, during situations of extended fracture healing, long-term bedrest, physical immobilization, and related conditions. In a broader sense, the data speak to the response of bone and mineralized vertebrate tissues to changes in gravitational loading and applied mechanical forces in general.

INTRODUCTION
The biological process of calcification involves the deposition of an inorganic mineral phase in the tissues of living organisms. Calcification is comprised of a series of different events and is complex, regulated in part by the activities of specialized cells, by the various properties of organic constituents found both within and outside such cells, and by specific physical and chemical interactions between certain of the organic components and the mineral (Glimcher, 1976, 1984). The five major calcified tissues of the vertebrates are bone and calcified cartilage of the skeleton and dentin, enamel, and cementum of the dentition; tendon normally calcifies in the skeleton of some vertebrate species, most notable among the avians. All these tissues share a number of features, including the fact that the bulk of the inorganic mineral phase is deposited extracellularly and is strictly ordered by the organic matrix (Christoffersen and Landis, 1991). About two-thirds of the dry mass of vertebrate calcified tissues, including embryonic enamel, consists of inorganic salts principally containing calcium and inorganic orthophosphate ions, carbonate as the third most prominent component, and sodium, magnesium, citrate, potassium, chloride, fluoride and other ions additionally in lower amount. The remaining one-third dry mass is organic in nature, in which cells constitute only a very small fraction of the total volume or mass of the tissues and the structural fibrous protein, collagen, comprises approximately 85-90% of the respective extracellular organic matrices in all but enamel among the major calcifying tissues including tendon. Amelogenins and enamelines, proteins chemically and structurally distinct from collagen, are the major organic matrix constituents in the enamel.

Less prominent in mass or volume than collagen, amelogenins, or enamelines, numbers of other matrix molecules are present in the vertebrate calcifying tissues in general. These include additional acidic proteins and phosphoproteins, proteoglycans, glycoproteins and sialoproteins, and phospholipids (Boskey, 1989). Interactions of each of these organic molecules with mineral ions occur and many details of their associations are well described while other interrelations can be considered only as hypothetical. One of the principal tenets of calcification in all the vertebrate tissues other than enamel, otooliths, the egg shell, and a few additional exceptions is that the ultimate repository of the mineral is collagen (Glimcher, 1976, 1984; Christoffersen and Landis, 1991). As may be illustrated by electron microscopy of the extracellular milieu of bone (Figure 1) or the other collagenous tissues, the collagen-mineral relationship is very specific and underscores the importance of the organic matrix in the calcification process.

With regard to the latter point, the various organic matrix components are thought to be functionally critical in calcification in that they mediate or control fundamental events by one or more of three common mechanisms (Glimcher, 1976). The first is through cellular activity leading to changes in local pH or increases in mineral ion concentration, possibly initiated by the action of specific enzymes such as alkaline phosphatase or by transport and release of calcium and phosphate ions involving extracellular matrix vesicles, mitochondrial granules, or, again, alkaline phosphatase (Glimcher, 1976; Boskey, 1989). The second mechanism for controlling calcification is through the generation of nucleation sites for mineral deposition. This may be accomplished by certain molecular groups comprising the extracellular organic constituents and capable of binding calcium and/or phosphate ions. These include, for example, phosphorylated amino acid residues of serine, threonine, or others (found in osteoponitin, bone sialoprotein, dentin phosphophoryn, bone acidic glycoprotein-75, and collagen among other molecules); e-aminogroups of lysine or hydroxyllysine (collagen); carboxyl groups of glutamic or aspartic acids (collagen) or γ-carboxyglutamic acid (ostecalcin); carboxyl or sulfate groups of hexose or hexosamines (proteoglycans); carbonyl oxygens of glycine (collagen); and phosphatidylserine or phosphatidlyinositol (phospholipids) (See Glimcher, 1976, 1984, 1989; Boskey, 1989; Veis, 1989; Gorski, 1992, for reviews and details). The third means for controlling mineral deposition is through the removal or degradation of inhibitors to
calcification. In this instance, the organic species involved may be proteoglycan molecules, sialo- or glycoproteins, or other components (Glimcher, 1976, 1984; Boskey, 1989).

As noted above, in most vertebrate calcifying tissues, the collagen-mineral association is the most prominent and it is highly specific and basic in the control of mineral formation. Because of its recognized importance, the interaction between these two components has been investigated most rigorously and many of its physical, chemical, and biological aspects have been described (Reviewed in Glimcher 1976, 1984, 1989; Veis, 1989). On the other hand, uncertainties exist with respect to a number of considerations, including in particular questions relating to three-dimensional (3D) atomic, molecular, and macromolecular levels of collagen-mineral structural hierarchy (Landis et al., 1993; Ziv et al., 1996). This paper summarizes results addressing such concerns and will present data from recent and current studies utilizing a number of different specimens as models for vertebrate calcification, analyzed with a variety of standard as well as novel experimental approaches. Some detail will be given of high voltage electron microscopic tomography and image reconstruction methods as applied to normal bone and calcifying tendon, the work leading to the first direct visualization in 3D of mineral crystals as they are observed in association with collagen in situ.

With respect to such collagen-mineral interaction, other data will be presented concerning effects on collagen observed in cultures of osteoblasts exposed to spaceflight. In this situation, an adaptation of the protein to a change in gravity suggests an explanation for bone loss by humans and other vertebrates subjected to weightlessness and to correlative conditions of skeletal unloading. The results detailed here provide a more complete understanding of
matrix-mineral events describing mechanisms that are critical in biological calcification, and they also lend insight into means by which calcified tissues respond to changing gravitational loads specifically and applied mechanical forces generally.

**MATERIALS AND METHODS**

Certain experiments cited below have utilized the long bones (tibiae and femurs) from normal embryonic (8-17 days old) chickens and the calcifying leg tendons (Achilles or gastrocnemius) from normal young (14-17 weeks old) domestic turkeys. The animals were sacrificed as described previously (Landis et al., 1977; Landis, 1986) and the tissues dissected immediately. Bones and tendons were prepared for electron microscopy by either of two procedures, a conventional methodology using glutaraldehyde or glutaraldehyde-paraformaldehyde fixation in aqueous solvents or an anhydrous technique employing treatment with 100% ethylene glycol. As applied to calcified tissues in this laboratory, each of these methods has been published (Landis et al., 1977) and the anhydrous treatment has been documented to maintain mineral in the tissues in an optimal fashion (Landis and Glimcher, 1978).

Morphological features of the embedded tissues were examined by light microscopy in 1 μm sections stained with toluidine blue and thin (~80 nm) or thick (0.25-0.5 μm) sections for electron microscopy were subsequently obtained by ultramicrotomy. Some of these sections were stained with uranyl acetate and lead citrate or left unstained. Thin sections were observed at 60-100 kV in the transmission mode of either a JEOL 100C or Philips EM 300 electron microscope. Selected area electron diffraction of mineral deposits in unstained sections was performed at 80-100 kV; gold was used as a standard to calibrate the diffraction measurements (Landis and Glimcher, 1978). Thick sections were studied by high voltage microscopy in the Albany, NY, AEI-EM7 instrument, operated at 1.0 MV. All conventional and high voltage microscopy employed liquid nitrogen to minimize section contamination and mass loss resulting from electron beam irradiation.

Some thick sections were analyzed by tomography and 3D image reconstruction techniques. In these cases, areas of interest were photographed at 10,000 - 12,500 x magnification in 2° tilt increments over an angular range of ±60° using a high tilt angle specimen holder. Photographic negatives were scanned optically in an Eikonix EC 78/99 or Vextel VX3000 image scanning system, and recorded images were stored in a Silicon Graphics Indigo2 XZ workstation or a Digital Equipment VAX 11/750 or 6210 interfaced with a Digital Equipment VT100 or VT125 monochrome graphics terminal and Lexidata 3400 video monitor. Images were aligned and manipulated with Advanced Visual Systems (Cambridge, MA), Voxel-View (Vital Images, Inc., Fairfield, IA), or SPIDER software (Wadsworth Center for Laboratories and Research, Albany, NY; Frank et al., 1981). The images were photographed with a 35 mm camera from the display screen of the Silicon Graphics workstation or a PIXAR graphics processor dedicated for 3D reconstructions and linked to a Sun Microsystems workstation. The complete system of consecutive image frames was also used to generate video recordings on cassette tape so that changes in structural features could be visualized in simulated variable motion as a function of depth through a given section of tissue. Individual frames of the tape were themselves used for obtaining information concerning spatial relations between collagen, mineral, and other structural components of tissue matrices. This approach has been described in detail (Frank, 1992) and applied in a number of calcification studies over the past few years (McEwen et al., 1991; Landis, 1995; Landis et al., 1993, 1996a, 1996b).

Other experiments have utilized a model calcifying system of primary osteoblast cell cultures derived from normal 14-17 day old embryonic chicken calvaria. The cultures have been extensively characterized under normal gravity (1g) in terms of their reproducible cell growth and development, extracellular organic matrix production, and mineralization (Gerstenfeld et al., 1988, 1989, 1993; Yang and Gerstenfeld, 1996). The cultures have also been studied (Landis et al., 1994, 1995) to understand whether such bone cells would adapt to changing gravitational conditions, in this case the environment of spaceflight presented during the 11-day mission of STS-59 (April 9-20, 1994). In this protocol, aliquots of embryonic chick bone cells (~7 x 10⁵), grown in Dulbecco's modified Eagle medium + 10% fetal bovine serum, were mixed with microrodger beads, inoculated into cartridge culture units composed of artificial hollow fiber capillaries (Cellico Cellmax Quad units; Cellico, Inc., Germantown, MD), and placed in a mid-deck locker aboard the shuttle, Endeavour (Landis et al., 1994, 1995).

In order to promote cell differentiation, cartridge media were supplemented with 12.5 μg/ml ascorbate and 10 mM β-glycerophosphate for varying time periods before and during flight. Four cartridges contained cells from 17-day old embryos grown for 5 days in the presence of ascorbate prior to launch (defined as flight cells committed to the osteoblastic lineage) and four cartridges supported cells from 14-day old embryos grown for 10 days with ascorbate prior to launch (uncommitted flight cells). Eight cartridges prepared in the same manner were maintained under normal gravity throughout the flight (control cells) and four additional identical cartridges under normal gravity were terminated on the day of launch (basal or pre-flight cells). From shuttle launch to landing, all cartridges were contained in closed hardware units maintaining 5% CO₂, 37°C, with delivery of fresh media at a rate of ~1.5 ml/6 hr. During day 3 and 5 of flight, duplicate aliquots (~3 ml) of conditioned media and accumulated cell products were collected in both the flight and control hardware units. At the termination of the mission, comparisons among basal, flight, and control samples were made in cell metabolism; gene expression for type I collagen and osteocalcin; and ultrastructure (Landis et al., 1994, 1995).
RESULTS AND DISCUSSION

The microscopic and imaging methods described above have yielded a large amount of structural information concerning the interaction of collagen and mineral in bone and other calcifying vertebrate tissues. For the presentation in this paper, the data will be summarized briefly with appropriate references to published work from this laboratory and elsewhere. In a similar manner, results from spaceflight experiments will be detailed. For clarity, certain observations will be given greater discussion.

The mineral and crystals of bone and calcifying tendon. Among the majority of vertebrates, the mineral form in mature tissues, including normal bone and tendon, is a salt principally composed of calcium phosphate, having the basic formula of Ca$_{10}$(PO$_4$)$_6$(OH)$_2$, hydroxyapatite or, more properly, apatite or dahlite, so-named because of a naturally small percentage (3-5%) of carbonate ion substitution for either phosphate or hydroxide ions (Lowenstam and Weiner, 1989). The precise structural nature and chemical composition of the mineral during its maturation and aging are not well characterized and, as mentioned earlier, other ions besides carbonate may substitute at either the cationic or anionic sites in the crystal lattice. Identity of the apatite lattice structure may now be approached with atomic force or other scanning probe microscopies (Siperko and Landis, 1992, 1993). Varying with time, the replacement of one ion for another, then, underlies in part both the complexity and uniqueness of the apatite. The mineral is novel in other aspects, including the presence of unusually small crystals having poor crystallinity, high surface area, and a defect structure incorporating ion substitutions, as noted, as well as lattice vacancies (Rey et al., 1990, 1991; Kuhn-Spearing et al., 1996). These factors significantly influence the functional processes in which the mineral is involved and undoubtedly impart in large measure the extraordinary degree of physical, chemical, and biological specificity found in vertebrate calcification. The crystals of apatite in bone, tendon, and other calcifying vertebrate tissues have minimum sizes of approximately 45 x 30 x 2-4 nm and principally take the shape of irregular platelets (Weiner and Price, 1986; Landis et al., 1993). Small angle x-ray scattering studies have provided the most recent size measurements (Fratzl et al., 1991) and electron diffraction has shown that the crystallographic c-axes appear to follow the longest crystal dimension, corresponding to its [100] face (Moradian-Oldak et al., 1991).

The shapes of the crystals have been visualized directly by microscopy and 3D imaging techniques (Figure 2), the latter showing that there is a strict orientation of the crystals in the collagenous matrix of the tissues such that their [100] faces are all generally parallel (within ~20°) to each other (Landis et al., 1993). As observed following tomography, crystals of different sizes appear at any particular point in time, larger crystals apparently forming from fusion of smaller ones (Landis et al., 1993). At least during early stages of crystal development, crystal growth occurs preferentially in the c-axial direction (lengthwise) with other growth present in the crystal width but not thickness (Landis et al., 1993). These features are important with regard to the structural organization of collagen and to the interaction between collagen and the apatite crystals, to be discussed below.

Collagen. Structurally, collagen comprises nearly all the organic matrices of bone, calcifying cartilage and tendon, dentin, and cementum. Mineralization occurs principally in association with type I and II collagen, the latter predominantly constituting calcifying cartilage, the former in the other tissues given above. These two collagen species, among the 19 now known (Adams, 1993; Fukai et al., 1994; Yamauchi, 1995), have been extensively characterized and consist of 3 polypeptide (α) chains, each about 1000 amino acids in length and containing glycine, proline, and hydroxyproline as compositional hallmarks. The 3 α-chains are arranged helically to produce a rod-shaped molecule approximately 300 nm in length and 1.2 nm in diameter (Lees, 1987). These molecules polymerize or aggregate in 2D in a highly specific manner through unique chemical crosslinks that dictate a lateral staggering of adjacent molecules about 1/4 to 1/5 of their 300 nm length (Hodge and Petruska, 1963). This orderly arrangement of type I or II collagen creates so-called hole (~40 nm in length) and overlap (~77 nm in length) regions in the packed arrays, together giving rise to a 64-70 nm periodicity characteristic of this protein (Hodge and Petruska, 1963). The hole and overlap sites accommodate the nucleation of mineral crystals and their c-axial development (Hodge and Petruska, 1963; Katz and Li, 1973a, 1973b; Glimcher, 1976; Arsenaault, 1991; Maitland and Arsenaault, 1991; Christoffersen and Landis, 1991; Landis et al., 1993, 1996a; Yamauchi and Katz, 1993; see below). In 3D, the assembly of collagen appears also to be highly ordered, again by particular chemical bonding (Fraser et al., 1983; Yamauchi et al., 1989; Yamauchi, 1995), such that the hole and overlap zones of adjacent collagen molecules and their 2D arrays are in very close register, thereby creating channels or gaps through the 3D assemblage (Hodge and Petruska, 1963; Katz and Li, 1973a, 1973b; Glimcher, 1976; Fraser et al., 1983; Christoffersen and Landis, 1991; Landis et al., 1993; Yamauchi and Katz, 1993). The space thus produced may accommodate crystal growth in width (Weiner and Traub, 1986; Traub et al., 1989; Landis et al., 1993, 1996a). A schematic of collagen molecular assembly in this manner is presented in Figure 3.

Collagen-mineral interaction. As just noted, specific regions of space, indeed certain resident molecular sites defined most likely by stereochemical and electrostatic charge distribution and other possible considerations, within type I and II collagen assemblages (and along collagen surfaces, as described below) are responsible for nucleation, growth, and development of apatite crystals in most calcifying vertebrate tissues. This view has been supported by recent observations of matrix-mineral spatial relations in normal bone and calcifying tendon determined for the first time by
Figure 2. A series of 6 tomographic surface-shaded renderings of a small, newly deposited crystal cluster located in a 0.5 μm thick section from the normally calcifying leg tendon from a young (15-17-week old) turkey (Landis et al., 1993). The collagen matrix associated with the crystals has been removed computationally in these image reconstructions. The views have been consecutively rotated through 25° and show that the composite crystals are irregularly shaped platelets, some having lengths and widths reaching 170 and 45 nm, respectively, in this particular volume. Thickness (~6 nm) appears relatively uniform among the platelets. The crystals are closely parallel to one another and are oriented such that their crystallographic ε-axes, along the crystal length dimension (thin arrow), are also parallel to the long axis of collagen (in the direction of the heavy arrow). Additional details of this and related tomographic reconstructions may be found in Landis et al. (1993). Magnification 105,000 x; bar = 0.1 μm.

electron microscopic tomography and 3D image reconstruction methods (McEwen et al., 1991; Landis, 1995; Landis et al., 1993, 1996a, 1996b). These studies, corroborating some results of earlier mineralization investigations based on conventional microscopy (Robinson and Watson, 1952; Nylen et al., 1960; Glimcher and Krane, 1968; Landis et al., 1977; Weiner and Traub, 1986, 1989; Traub et al., 1989, 1992; Arsenault, 1991; Maitland and Arsenault, 1991; Moradian-Oldak et al., 1991), chemical crosslink analysis (Yamauchi et al., 1989, 1993; Yamauchi, 1995), diffraction techniques (Nylen et al., 1960; White et al., 1977; Berthet-Colominas et al., 1979; Fraser et al., 1983; Moradian-Oldak et al., 1991), and other approaches (Katz and Li, 1973a, 1973b; Weiner and Price, 1986; Fratzl et al., 1991) have documented many aspects of matrix-mineral interaction, including the following: [1] The shapes of early crystals are those of irregular, thin platelets, ~45-170 x 30-45 x 4-6 nm in size; [2] crystal nucleation occurs at multiple spatially and temporally independent sites both on the collagen surface and within collagen in its hole and overlap regions; [3] of the crystals located within collagen, hole zone nucleation is the predominant event compared to overlap nucleation at the earliest stages of mineralization; [4] newly forming crystals grow preferentially in length, corresponding to their crystallographic ε-axis; [5] crystals would appear to grow in their width along the channels or gaps formed by adjacent collagen hole regions in register; [6] smaller crystals may fuse in a coplanar alignment to produce larger crystals and such development may exceed the dimensions of single collagen hole zones; [7] the ε-axes of respective crystals are in general parallel (within ~20°) to one another and to the long axis of the collagen fibrils with which they associate; [8] adjacent crystals are separated by
Figure 3. A schematic diagram of the assembly and mineralization of collagen molecules in 3D, deduced in part from tomography and image reconstruction and following the description given in Landis et al. (1993). Individual molecules of collagen are shown as narrow cylinders 300 nm in length and 1.23 nm in diameter (Lees, 1987), crosslinked in 2D in the quarter-staggered pattern defined by Hodge and Petruska (1963). Dimensions of the hole and overlap regions of the fibrils are 40 and 27 nm, respectively, and the space between adjacent molecules is 0.24 nm (Lees, 1987). Packing of molecules in 3D creates hole zone channels or gaps (illustrated by heavily outlined rectangular boxes in the second left drawing of the figure) as a result of the strict registry of hole and overlap sites among molecules. Shown in the second right drawing of the figure with 3 layers of molecules, early crystals nucleate at independent sites principally in the hole regions of the collagen arrays and the platelets become oriented during the process. Progressive development of the individual crystals (far right drawing) occurs in which there is preferential growth in the c-axial length of the platelets following the long axis of the collagen molecules and growth in width of the platelets along the extensive hole zone channels. Smaller crystals abutting one another in local volumes of the arrays may fuse to form larger platelets that remain generally coplanar and in approximately the same orientation with respect to one another and to collagen. A series of thin, still irregularly-shaped, roughly parallel sheets of larger and larger crystals would ultimately occupy the collagen assemblages. Defined crystals that are unstaggered with respect to one another are separated by ~4.2 nm, sufficient space for a packing arrangement conceptually accommodating 3-4 collagen molecules. This diagram is reprinted with permission from the Journal of Structural Biology.

~4.2 nm, a space sufficient to accommodate 3-4 layers of collagen molecules packed in a specific manner, and [9] crystals located in different collagen fibrils may be coherent in their orientation and alignment so that their organization is highly uniform over large matrix volumes. A number of these points are incorporated in the schematic of Figure 3 and the image reconstruction of Figure 4.

This current view of collagen-mineral interaction is very useful in understanding facets of the basic mechanism(s) of vertebrate mineralization, but there are other areas of study to be addressed in order for information to be still more instructive. Among these are questions
regarding the precise structural, stereochemical, or physical-chemical environment of the hole and overlap regions and the surface of collagen and how it mediates platelet nucleation and subsequent growth; the possible role of other collagen species and the non-collagenous proteins in type I and II collagen assembly and mineral deposition; the manner in which progressive development of coplanar, generally parallel crystals at a molecular and macromolecular level of structure occurs at supramolecular, morphological, and higher orders of structure among the vertebrate calcifying tissues; and the relationship between such organization and its consequences in terms of tissue integrity, strength, and biomechanical properties. Some of these issues are under active investigation in many laboratories and forthcoming data will hopefully provide greater insight into the fundamental collagen-mineral associations and other events in vertebrate calcification.

**Skeletal Adaptation to Gravitational Loading.** It is interesting to consider whether changes in the collagen-mineral interaction manifest themselves in the adaptive capacity of the skeleton in response to a shift in loading or unloading. External environmental forces, including gravity, buoyancy, or other applied mechanical effects, are known to result in adaptation by the skeletal system of the vertebrates (Lanyon, 1992; Jee and Frost, 1992; Biewener and Bertram, 1993; Burger and Veldhuijzen, 1993; Morey-Holton et al., 1996; Carmeliet et al., 1997). Tissue structure is altered through its architectural geometry or...
Figure 5. Northern blot analysis of steady state levels of collagen (Col 1A1) and osteocalcin (OC) mRNA expression in flight (F) and ground control (G) cartridges carrying embryonic osteoblasts during the STS-59 shuttle mission. Data presented are for cells derived from cultured calvaria of normal 14-day old embryonic chicks (Uc, denoting cells uncommitted to the osteoblastic lineage); cells from 17-day old embryos (committed to the lineage) were also analyzed (Landis et al., 1994, 1995) but data are not shown. The integrity of the total RNA from the cartridges is given by the ethidium bromide-stained gels (EtBr) (upper and lower bands are 27S and 18S rRNA, respectively) and the loading of the lanes was determined and verified by analysis of 18S rRNA alone. The graphic analysis following normalization of flight and control gel bands to respective 18S rRNA depicts quantitation of the relative amounts of type I collagen and osteocalcin expression. Expression was determined as flight percent of ground control. Analysis was carried out by scanning densitometry of two separate blots developed from the RNA isolated from individual cartridges. Error bars represent the total range of variation (± one standard deviation) of the relative RNA quantities measured in 3 replicates within each of the two blots. The data show that, normalized on a per cell basis, the mRNA expression of both type I collagen and osteocalcin from flight cells (uncommitted to the osteoblastic lineage) is reduced about 45% compared to control (1g) cells. Other data indicate that, following the same analytical procedure, expression of type I collagen and osteocalcin from flight cells committed to the osteoblastic lineage is reduced 50% and 55%, respectively, on a per cell basis compared to controls (Landis et al., unpublished).

form, mass, or composition, and the skeleton as a whole may be modified in development, metabolic state, and function (Jee and Frost, 1992; Burger and Veldhuijzen, 1993; Morey-Holton et al., 1996). These changes are thought to be mediated ultimately at the cellular level of structural hierarchy (Morey-Holton et al., 1996), and, in this context, a characterization following spaceflight of the cultures of normal embryonic chicken bone cells described previously is particularly relevant.

Following the STS-59 mission, it was found (Landis et al., 1994, 1995) that both the committed and uncommitted flight cells were metabolically active, as measured by glucose uptake and lactate production, at approximately the same statistical levels as control counterparts. Data analysis also showed that the flight cells elaborated a less extensive extracellular matrix, evidenced by an approximately 45-50% reduction in collagen gene expression (normalized to a per cell basis; Figure 5) and a correlative collagen protein appearance (Landis et al., unpublished) compared to control cells. Osteocalcin was expressed by all cells, a result indicating progressive differentiation of both flight and control osteoblasts, but its message levels, like collagen, were also reduced (~45-55%) in flight cells compared to ground samples (Figure 5). This finding of diminished osteocalcin gene expression suggests that osteoblasts subjected to flight follow a slower progression toward a differentiated function than their counterpart controls maintained at 1g. The summary of data indicates that spaceflight, including microgravity exposure, clearly affects bone cells by down-regulating type I collagen and osteocalcin gene expression. An inhibition in expression of the osteogenic phenotype (notably by committed osteoblasts) follows as does a reduction in collagen synthesis and secretion. The information is important for more complete insight into the basic biology, biochemistry, and physicochemistry of the adaptive events in bone, and particularly with reference to spaceflight.

The down-regulation of type I collagen and osteocalcin gene expression and a slowing of osteoblast
progression toward a differentiated function are measures of cellular control found in the spaceflight experiments described and documented in part in Figure 5. It appears, then, that flight exerts effects on the osteoblasts themselves, a result supporting the concept, earlier noted, that cells sense environmental change and dictate a response to it. Cellular regulation at the gene level in this manner by osteoblasts would be expected to limit collagen secretion and synthesis, leading, at least in part, to a reduction in bone mass through collagen-mineral interaction discussed earlier. Possible alterations in bone composition, geometry, and other consequences could also occur. This result may be especially critical in considerations of bone adaptation to forces in general, as just stated, to bone loss experienced by humans and other vertebrates during spaceflight, or in cases of prolonged fracture healing, long-term bedrest and immobilization, and related conditions in normal gravity. The information here may also have significant implications regarding bone maintenance against the effects of osteoporosis and other bone degenerative pathologies. Advancing the types of experiments described above with model culture systems flown in space should provide greater insight into the adaptive nature of osteoblasts and those other cells that mediate a calcified matrix in the vertebrates. Moreover, extrapolation of the present experimental design to situations of hypergravity may yield additional valuable data. Preliminary results obtained from the latter indicate changes in cellmetabolism, gene expression, and cellular and extracellular matrix ultrastructure (Kacena et al., 1998; Landis et al., 1998).

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