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Perspective Article

Hylonome

Aging and protein expression

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Aging

A recurrent debate within gerontology is whether aging is a disease or a byproduct of a normal process. While increased age is associated with the increased presentation of pathologies, the constellation of changes that do occur with advanced age reflect organ system wide alterations and a personal heterogeneity of expression that are more consistent with a process than a disease. On a given organ system level, individual age-associated pathologies have been identified (for example, hypertension for the cardiovascular system and bone loss for the skeletal system) but whether there is a systemic disease that is aging is a matter of perception. The definition of aging as a disease depends as much on research priorities, pharmaceutical and funding opportunities as it does on clinical nosology. A relevant framework to view aging from is one where aging is viewed as a breakdown of homeostasis, or maintenance of specific molecular pathways. This breakdown is the inevitable consequence of the evolved anatomical and physiological design of the organism. Examples of maintenance pathways include DNA repair, synthesis fidelity, defense against oxygen free radicals, wound healing, immune response and physiological homeostasis.

Bone loss

The most visible and functional evidence that the aging process has left its imprint on our bodies lies in the musculoskeletal system. The elderly appear to have lost height, their posture is bent in part due to the development of kyphosis, and a cane provides the support and security that are required because of weakened musculature and peripheral nerve degeneration. These reflect the fact that significant changes have occurred in all the components of extracellular matrix: in ligaments and tendons, in muscle fiber composition and in the structure and quantity of bone mass. As a result of these alterations in extracellular matrix composition, the elderly typically display thinned skin that is more susceptible to injury, diminished muscle bulk that results in weakness that magnifies the effects of neurologic degeneration on gait and posture, and diminished bone mass that increases the risk of fracture.

The loss of maintenance of bone mass that is associated with aging has multiple foci. Because bone homeostasis involves the finely tuned balance between bone formation and resorption, events/factors that perturb that balance are viewed as causal. For the endocrinologist, hormonal changes (estrogen for women, growth hormone/IGF-I for men) are central. For the molecular biologist, the genetic program and its temporal interaction with the environment lead to altered gene expression and imbalance. For the cell biologist, changes in the cytokines involved in the coupling system are key. For the modern biologist, the choice between apoptosis or maintenance as well as cell recruitment to the adipocytic or osteoblastic lineage are fruitful starting points. For a protein chemist, the changing composition of the extracellular matrix is fundamental as the matrix provides not only structural integrity, but also the modifying environment in which hormones, growth factors and cytokines are presented. From this perspective, the more distal, functional molecular consequences associated with bone loss are investigated.

Collagenous and non-collagenous protein components of skeletal tissues

The structure and metabolism of the major structural protein of bone, type I collagen, is well known. Two pro-α1(I) and one pro-α2(I) polypeptide chains are processed both intra- and extracellularly such that self assembly of triple helical molecules into fibrils and then collagen fibers occurs. The collagenous network is stabilized by covalent cross-links within and between the alpha chains by the formation of somewhat bone-specific deoxypyridinoline cross links. The importance of type I collagen structure in bone matrix metabolism is reflected in observations of the pathophysiological conse-

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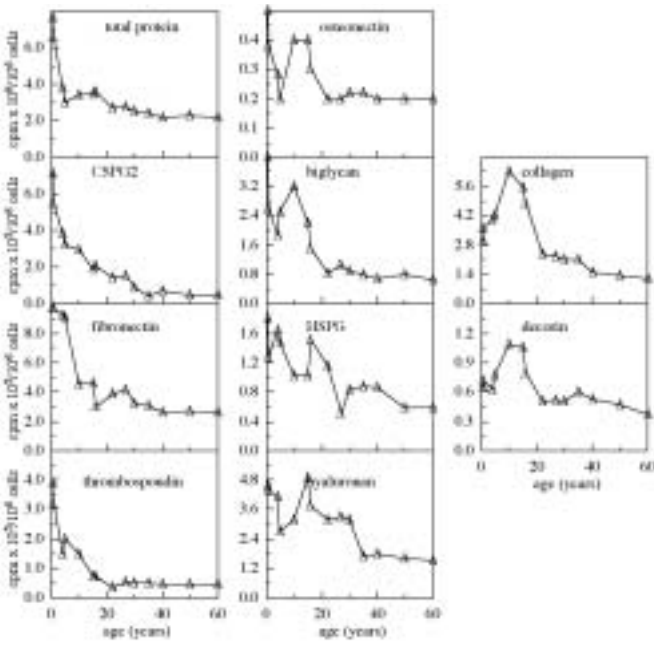

Figure 1: Patterns of human bone matrix component synthesis. Human bone cells derived from 22 donors of ages spanning fetal to 60 years were isolated and grown as explants. Seventy-two hours after passage to primary, the cultures were radiolabeled for 24h with [35S]sulfate and [3H]leucine/proline and the amount of label incorporation into various extracellular matrix components was determined and normalized on a per cell basis as described[11]. Three distinct patterns were observed. The first pattern was defined by high perinatal levels and an asymptotic decrease with increasing age. The second pattern exhibited high levels in fetal and peri-pubescent donors and the third pattern showed a peak around the pubertal growth spurt age range and low fetal and post-pubertal levels. CSPG2, versican-like proteoglycan; HSPG, syndecan-like proteoglycan.

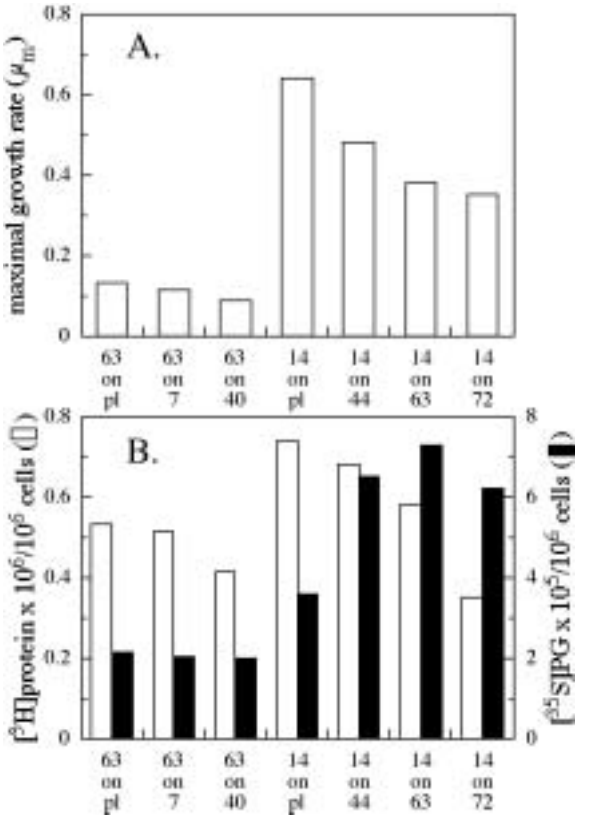

Figure 2: Effect of different donor age matrices on proliferation and synthesis. (A) Maximal growth rates were determined from crystal violet staining of 3 weeks of cultures and growth curves modeled by the logistic regression as described[36]. (B) Osteoblasts derived from a 63-year-old normal donor or a 14-year-old donor were passage to plastic culture dishes (pl) or dishes coated with the respective autologous "ghost" matrix or a "ghost" matrix from either young or old donors (ages 7, 40, 44, 63 and 72). Ghost matrices were generated by treatment of confluent cultures with 0.5% Triton X-100 in phosphate buffered saline for 20 min[55]. Cell removal was monitored by microscope. Plates were rinsed three times with phosphate buffered saline and stored at 4°C for up to two weeks prior to use. The level of [3H]proline incorporation into protein and [35S]sulfate incorporation into proteoglycan (PG) was determined as described[11].

quences of mutations in the genes that code for it that are the cause of the brittle bone disorder, osteogenesis imperfecta[1].

Bone contains at least four distinct proteoglycan types – a versican-like chondroitin sulfate proteoglycan, a syndecan-like heparan sulfate proteoglycan and the two small interstitial proteoglycans decorin and biglycan[2,3]. Decorin binds to ("decorates") the collagen fibrils[4] while biglycan is found in pericellular areas, including that of osteoblasts and osteocytes[5]. The small proteoglycans may be acting as matrix organizers, orienting and ordering the collagen fibrils, with the protein portion binding to collagen fibrils at specific sites and the glycosaminoglycans chains aggregating to hold the proteins and hence the collagen fibrils at defined distances from each other[6]. Targeted disruption of the biglycan gene yields a mouse phenotype that suggests that biglycan may be a molecular determinant that contributes to peak bone mass[7]. Although the exact function of another bone proteoglycan, versican, is not known, it has been proposed to act as a space filler in fetal and very young bone, to be replaced by osteoid later[8]. Hyaluronan (or hyaluronic acid) is found in bone[9]. It is synthesized by bone cells in culture and has been proposed to "capture space" for subsequent matrix deposition[2,10,11]. Within this context, hyaluronan is a component of "immature" bone matrix and its increased levels in bone matrix is consistent with the early, "immature" bone matrix being less structurally rigid compared to mature, highly collagenous matrices.

The recently coined SIBLING family (for small integrin binding ligand N-linked glycoproteins) include bone sialoprotein, dentin matrix protein 1, dentin sialophosphoprotein, matrix extracellular phosphoglycoprotein and osteopontin[12]. Bone sialoprotein is expressed at late stages of osteoblastic differentiation and found to mark cells in the secretory but not in the proliferative compartment, and has been localized to the sites of earliest sites of mineral deposition[13-16]. Immunolocalization of osteopontin revealed the highest density of gold particles associated with electron-dense organic material found at the mineralization front and in 'cement lines'[17]. Because of their inherent polyanionic nature, the sialoproteins can bind calcium and hydroxyap-

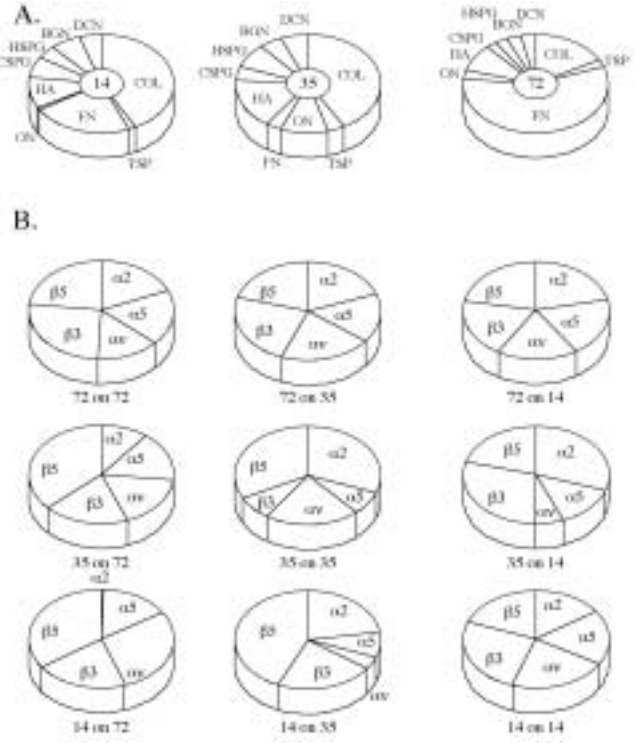

Figure 3: Effect of different donor age matrices on integrin expression. (A) "Ghost" matrices were generated from 14. 35 and 72-year-old donors and, in parallel cultures, matrix component synthesis was analyzed to determine the relative distribution of collagen (COL), thrombospondin (TSP), fibronectin (FN), osteonectin (ON), hyaluronan (HA), versican-like proteoglycan (CSPG), syndecan-like proteoglycan (HSPG), biglycan (BGN) and decorin (DCN). (B) Osteoblasts derived from 14, 35 and 72-year-old donors were passaged onto tissue culture dishes coated with "ghost" matrix. After 4 days in culture the cells were scraped in PBS, separated into five aliquots and incubated with either anti-alpha2, alpha5, alphaV, beta3, or beta5 integrin antibodies (R&D, Inc.) and subjected to FACS analysis using the Johns Hopkins Core FACS facility.

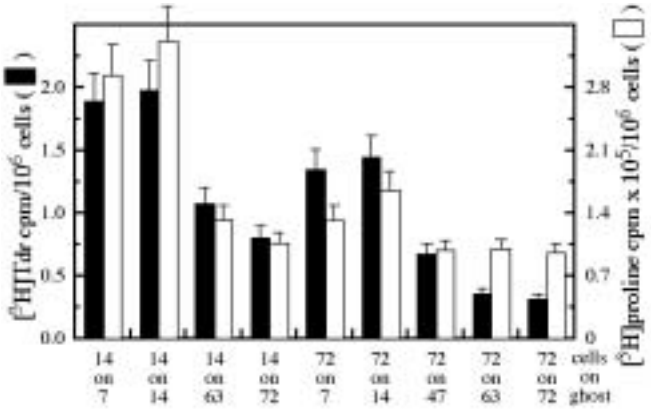

Figure 4: Effect of different donor age matrices on response to basic fibroblast growth factor. Osteoblasts derived from different age donors were seeded onto "ghost" matrices derived from either autologous cells or different age donors and after 24 hours, the effect of 100 nM basic fibroblast growth factor on proliferation (measure as [3H]thymidine uptake) and protein synthesis ([3H]proline incorporation) was determined after 12 hours.

atite - coating the mineral phase of bone and then, via their RGD moieties, enabling cellular adherence to specific sites. Mice lacking a functional osteopontin gene exhibit a phenotype of increased mineral content and crystallinity[18]. Targeted disruption of the bone sialoprotein gene has yet to reveal an apparent phenotype.

Osteonectin (also termed SPARC, culture shock protein or BM40) is Ca^{2+}-binding glycoprotein found in many connective tissues, though relatively high concentrations are present in bone[19]. Osteonectin inhibits hydroxyapatite crystal growth[20], and binds to collagens type I, II and IV, as well as to thrombospondin[21-23]. Targeted disruption of osteonectin/SPARC in mice yields normal development, a phenotype of severe cataract formation and low bone mass with increasing age[24].

Fibronectin and thrombospondins are RGD-containing proteins found in bone that are composed of various combinations and content of modular structures. They are believed to play a role in cell-matrix adhesion via binding to integrin receptors on the cell surface[25]. Thrombospondins possess distinct domains that modulate matrix protein interactions, cell attachment, migration and proliferation. Fibronectin is a multifunctional dimeric protein that also has the capacity to modulate cell migration, cell attachment and matrix organization. It contains binding domains for fibrin/heparin, gelatin/collagen and cell surfaces.

Osteocalcin comprises 10 to 20% of the non-collagenous proteins in bone, depending upon developmental age and the species. Levels of osteocalcin are low at early stages of bone formation and increase with increasing age. Targeted disruption of the osteocalcin gene in mice yielded a phenotype of increased bone mass and strength, suggesting that this small protein normally inhibits bone growth and promotes bone resorption[26].

Changes in skeletal matrix proteins with age

From the viewpoint of the human, with increasing age, changes in collagen are manifest as stiffening of joints due primarily to alterations in the normal intermolecular crosslinking of collagen molecules[27] and associated with loss of mineral density as manifested by increased fracture rate. Cross-linking of collagen molecules involves two independent mechanisms: the enzymatic mechanism involves oxidation of lysyl and hydroxy lysyl residues to aldehydes with the formation of divalent cross-links. A second mechanism termed glycation is non-enzymatic, and involves reaction with glucose and oxidation of the complex to form Amadori condensation products including furosine. These glycation cross-links alter the surface charge on collagen fibrils and lead to increased stiffness[28] and enhance osteoclast-induced bone resorption *in vitro*[29]. In human bone, the nonenzymatic

glycation products exhibit an age-related increase in levels[30].

As the levels of collagen cross-links increase with increasing age, the ability to extract intact collagen decreases. Aside from type I collagen, noncollagenous components such as the cell attachment proteins fibronectin, thrombospondin, osteopontin are also degraded to lower molecular weight fragments with advancing age[31]. It is not known whether the age-related degradation of these proteins affects bone cell function in aged individuals. A study of age-related changes in the composition of bone showed that the amounts present of alpha 2HS-glycoprotein, albumin, sialoprotein, soluble collagen and of EDTA-soluble protein were all higher in bone from children than in adults[32]. In the EDTA extracts, the collagen extractability decreases markedly with age and the sialic acid content increases[33].

With increasing donor age, human bone cells grown in culture exhibit reduced proliferation rates[11,34-36]. Osteoblasts from older donors produced less collagen, osteonectin, fibronectin, thrombospondin, hyaluronan, versican, decorin and biglycan with increasing donor age (Figure 1)[11,37]. The amount of collagen and the noncollagenous proteins incorporated into the extracellular matrix also decreased with increasing donor age[10,11]. Age-related changes in fibronectin structure and effects on osteoblasts have also been seen[38,39]. The reduction of matrix component synthesis, secretion and incorporation into the extracellular matrix has consequences not only for structural integrity, but also for signal transduction and feedback systems.

Matrix components such as thrombospondin[40], the syndecan-like heparan sulfate proteoglycan[41], as well as biglycan and decorin[42] have the capacity to bind to and alter presentation of growth factors and cytokines to bone cells. Signal transduction appears impaired in a number of systems during aging. The classical example is the generalized decrease in adrenergic responsivity with aging observed in multiple cells and tissue type age-related diminution of the adrenergic system[43]. The mitogenic response of osteoblasts to hormones and growth factors appears blunted in cells from aged donors[44]. The responsiveness of osteoblasts derived from older donors to IGF-I and estradiol were significantly reduced when compared to the responsiveness of cells derived from younger donors[45,46]. IGF-I receptor levels remained unchanged, while estrogen receptor alpha levels increased with increasing age.

Age-related changes in the signaling initiated by members of the Transforming Growth Factor-β (TGF-β) superfamily have also been observed. This multigene family includes the TGF-β isoforms, the activins, inhibins, Mullerian inhibitory substance, growth differentiation factors and several bone morphogenetic proteins (BMPs)[47]. An age-related diminution of TGF-β has been observed in bone[48]. The rate and quantity of ectopic bone formation in response to bone morphogenetic protein are reduced in aged rats, and suggest that the difference in blood vessel distribution is related to this reduction in ectopic bone formation[49].

Signal transduction does not appear to always be diminished with increasing age. Members of the interleukin family (IL-1, IL-6, IL-11), the tumor necrosis factors (TNF), macrophage-colony stimulating factor (M-CSF) and prostaglandins E2 and F2 also play important roles in modulating osteoblast and osteoclast activity[50]. An age-related decrease in PGE2 signaling through its receptor has been observed[51]. However, for IL-1, IL-6 and TNF alpha (and other inflammatory proteins such as C-reactive protein), aging is associated with increased circulating levels reminiscent of a chronic inflammatory state[52,53]. Expression of these factors by bone cells were also elevated[54]. The increases in these cytokines are believed to favor resorption.

Biological consequences of altered matrix composition

We have recently been studying the effects of altered extracellular matrices on cell proliferation and protein expression *in vitro*. Altered matrices can be generated by mixing and matching osteoblast cells derived from donors of various ages to isolated "ghost" matrices[55] from the same cell strains. In addition, the effect of pure, recombined extracellular matrix components in different stoichiometries has also been studied. Switching bone cells derived from young donors to growth on ghost matrices from cells derived from older donors lead to an altered protein expression pattern and growth rate (Figure 2). Cell surface receptors for matrix components, such as integrins also changed dependent on the matrix substrate, though cells derived from older donors did not exhibit the same magnitude of response as those derived from younger donors (Figure 3). In the purified matrix component system, the proliferative capacity of osteoblasts was found to be directly altered by changing the amount of type I collagen present in the culture matrix. The addition of matrix modifiers, such as fibronectin or thrombospondin, also caused an altered cellular metabolism. In addition, the responsiveness of bone cells to TGF-β, basic fibroblast growth factor and other cytokines was different depending upon the source and composition of the extracellular matrix (Figure 4). These observations point to the fundamental importance of the extracellular matrix in maintaining bone cell metabolism and modulating feedback from the extracellular environment to the cell. The consequences of an altered extracellular matrix composition have profound implications for prospective gene and stem cell therapy where the host microenvironment may override the therapeutic intervention.

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