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EDITORIAL

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"True bone" in vitro?

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The article by Ogose et al. [18] published in this tissue of *Virchows Archiv* pursues as its sole objective the study of the conditions under which cultured human bone cells are able to form bone in vitro. At first glance this seems to be a commonplace question that hardly deserves such consideration. Why, then, does it merit attention?

The model is attractive. Cultured human bone cells which behave in a manner analogous to their behavior in vivo may reflect functional disturbances of bone formation (osteogenesis imperfecta, osteoporosis, osteomalacia) whose causes can be investigated convincingly in the living model, using the methods of cell biology in ways that give more information than biochemical, molecular genetic, or morphological studies on bone biopsies alone. In addition, experiments concerning the stimulatory effects of mediators, cytokines, and drugs can be performed, as can studies on the biocompatability of heteroimplants (bone replacement, prosthetic surfaces).

All this, however, only makes sense if the in vitro behavior of human bone cells matches that of human osteoblasts in vivo. The examination of this question includes the application of stringent scientific criteria which have been developed since the beginning of the reliable isolation and cultivation of human bone cells [3, 9, 33] and are also used for osteosarcoma cell lines [5, 12, 21, 23, 25, 29]. Isolated bone cells (osteoblasts, osteocytes, medullary stromal cells) must possess at least four qualities in order to be classifiable as osteoblast-like cells: they must produce collagen type I (COL I), they must be able to form alkaline phosphatase (AP) and the non-collagenous bone protein (NCP) osteocalcin (OC, bone Gla protein), either spontaneously or under stimulation with $1,25(OH)_2D_3$, and they must respond to parathyroid hormone (PTH) with a cAMP increase [1, 9, 13, 22]. Fulfillment of further criteria, such as formation of the NCPs osteonectin (ON), osteopontin (OPN), bone sialoprotein (BSP), decorin (DCN), and biglycan (BGN), is not considered necessary for the phenotype to be described osteoblastic, since these proteins can also be formed by other cells [8, 31].

The determination of protein formation has been sufficient to allow the cell biological definition of the osteoblast in vitro. The time of this determination has been advanced to the earlier differentiation level of mRNA expression for bone-specific proteins using molecular biological methods [19, 26]. The visible deposition of bone matrix, although regarded as the culmination of in vitro differentiation, is not considered absolutely necessary for demonstration of osteoblast function, probably because it has not been possible to achieve definite in vitro deposition of bone matrix with mature human bone cells from adult donors, although numerous osteosarcoma cell lines and isolated normal bone cells have been shown to form the chief matrix proteins of bone and to be able even to produce ectopic bone formation in diffusion chambers after implantation into nude mice [14]. In vitro, however, problem-free and immunohistochemically or electron microscopically demonstrable production of a mineralizing matrix containing collagen fibers is currently only possible when fetal cells (mouse, rat) are used [2, 4, 6, 7, 10, 16, 28, 34]. In accordance with our own observations, previous reports on the formation of a mineralized matrix by human bone cells in vitro do not withstand critical examination, since only staining for light microscopy (von Kossa's stain) was done to demonstrate the mineralized matrix (and thus only mineral deposits), and the necessary verification of in vitro deposition of collagen fibers by electron microscopy did not take place.

Ogose et al. [18] start their approach to the problem from this point. By adding ascorbic acid and glycerophosphate to their cultures, they have identified this form of in vitro mineralization as dystrophic calcification, a matter of mineral deposition into necrobiotic cells or the residues of such cells. Under identical conditions, this deposition can also be achieved with other cells that do not originate from bone, especially if these cells are able to form the enzyme AP, as has been shown for a gastric

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carcinoma cell line [18]. For this reason, mineralization alone can on no account be taken as evidence for osteogenic potency in vitro [14, 18].

Searching for suitable conditions for the formation of true bone matrix by adult human bone cells (and by osteosarcoma cell lines) in vitro, using the observations reported to date in order to establish a reliable cell culture model of the "osteoblast-matrix unit", is logical and in line with the ideas and the previously futile attempts of many bone cell researchers. Successful induction of a mineralized bone matrix in vitro seems to be subject to the following important conditions: The medium must possess a sufficient supply of metabolically convertible components for bone matrix molecules, must contain ascorbic acid, and must have a calcium concentration corresponding to physiological conditions (α-MEM) with the addition of a low-dose phosphate ion source (5 mM β-glycero-phosphate). The culture period must exceed the "mineralization lag time" in vivo considerably [20] and amounts to more than 8 weeks for dense matrix mineralization in vitro. Excessive dosage of the phosphate ion source seems to lead to dystrophic calcification, which prevents the last differentiation step of cultivated bone cells associated with the production and extracellular deposition of a matrix that contains collagen fibers. Electron-microscopic control for mineralized matrix formation is necessary because only this control will provide information about the formation of a bone-like matrix. For this reason, electron microscopy should be demanded as a standard procedure for the demonstration of bone formation in vitro.

The approach taken by Ogose et al. [18] is important and provides a new platform for further substantiation and creation of an in vitro model of the osteoblast-matrix unit. But is the substance that is being formed really bone, or is it merely a "bone-like matrix" produced by "osteoblast-like cells? This question must now be refined: how far remote from "true bone" is the matrix formed and mineralized in vitro?

Bone matrix not only has a complex composition; its development is likewise complicated and apparently occurs only if the various constituents are produced, secreted, and deposited together extracellularly in the correct sequence. This demands sequential gene expression during the differentiation and activation of osteoblasts. In cell culture this expression starts with the mRNA transcription of COL I and AP and proceeds via mRNAs of OPN, BSP, DCN, and BGN to the transcription of the OC gene [17, 24, 26, 27, 32, 35, 36]. This sequential order also seems to determine the translation and release of the proteins. At the same time the matrix undergoes maturation for 10–15 days before mineralization sets in (mineralization lag time). During this time the NCPs (10% of the matrix protein content) are added to the COL I (90% of the protein content). The NCPs are not only bone-specific structural and mineralization proteins but also ligands for adhesion molecules and the storage site for matrix-bound latent proteolytic enzymes and bone growth factors. NCPs do not only originate from osteoblasts but are also secondarily bound to the matrix as serum proteins.

Table 1 Composition of proteins forming human bone matrix (*TPC* total protein content; data from [8, 11, 15, 30])

Structural bone matrix proteins	**Cytokines and growth factors**
Collagen I (85–90% of TPC)	Bone morphogenic proteins (BMP) 1–7
Non-collagenous bone proteins (10–15% of TPC)	Fibroblast growth factor (FGF)
	Insulin-like growth factors (IGF)
Thrombospondin	IG-binding proteins (IGF-BP)
Fibronectin	Transforming growth factor beta (TGF-β)
Biglycan	
Decorin	Platelet-derived growth factor (PDGF)
Bone sialoprotein	
Osteopontin	
Osteonectin	
Matrix-Gla protein	**Metalloproteinases**
Osteocalcin (bone Gla protein)	
α-2-HS glycoprotein	Latent collagenase

This unexpected and unique combination of structural proteins, bone induction proteins, and growth factors (Table 1), unknown only a few years ago, is the bridge leading to the understanding of the balanced function of bone formation and resorption (remodelling, coupling), fracture healing and bone induction, pathological remodelling processes, and structural alterations in hereditary and acquired bone diseases. The matrix-bound bioactive proteins, released when needed or through pathological processes, are part of an autocrine feedback mechanism of the bone cells which regulates the proliferation and differentiation of the osteoblasts. The proteins act as "chemo-attractants" for osteoclast precursors, as ligands for the adhesion molecules of these precursors, as activatable latent protease for the mature osteoclast, and as coupling factors for bone resorption and bone formation through the release of growth and differentiation factors for the osteoblasts [11]. To what extent this composition of "true bone" can actually be achieved in vitro must be one of the important questions of future research, because the answer to this question might provide a model for the elucidation of many bone diseases and also for the development of biologically suitable bone replacement materials.

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