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Developmental control of chondrogenesis and osteogenesis

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ABSTRACT During vertebrate embryogenesis, bones of the vertebral column, pelvis, and upper and lower limbs, are formed on an initial cartilaginous model. This process, called endochondral ossification, is characterized by a precise series of events such as aggregation and differentiation of mesenchymal cells, and proliferation, hypertrophy and death of chondrocytes. Bone formation initiates in the collar surrounding the hypertrophic cartilage core that is eventually invaded by blood vessels and replaced by bone tissue and bone marrow. Over the last years we have extensively investigated cellular and molecular events leading to cartilage and bone formation. This has been partially accomplished by using a cell culture model developed in our laboratory. In several cases observations have been confirmed or directly made in the developing embryonic bone of normal and genetically modified chick and mouse embryos. In this article we will review our work in this field.

KEY WORDS: *chondrogenesis, osteogenesis, angiogenesis, cell culture, growth factors.*

Introduction

During vertebrate embryogenesis bones of vertebral column, pelvis, and upper and lower limbs, are initially formed as a cartilaginous model subsequently replaced by bone. This process is called endochondral ossification. Endochondral ossification is characterized by a very precise series of events including aggregation of committed mesenchymal cells and differentiation of chondrocytes proximally located followed by hypertrophy of the same cells. Each stage of chondrocyte differentiation is characterized by modifications in cell proliferation, cell morphology, nature and amount of extracellular matrix macromolecule production. Collagen and proteoglycans are the major components of the extracellular matrix. In particular collagens II, IX, and XI and aggrecan are maximally synthesized by resting, proliferating and maturing chondrocytes, whereas type X collagen is synthesized by chondrocytes after they have become hypertrophic and before mineralization of the extracellular matrix occurs.

Bone formation initiates at the periphery of the hypertrophic cartilage core which is invaded by blood vessels and eventually replaced by bone tissue and bone marrow. After birth, cartilage remains in the growth plate of long bones and endochondral ossification continues until sexual maturity is reached.

In this article we will review our work on cellular and molecular events leading to cartilage and bone formation. In particular we will focus on mechanisms controlling stem and progenitor cell proliferation and differentiation, onset of chondrogenesis and fate of hypertrophic chondrocytes. Emphasis will be given to modulation of chondrocyte differentiation by growth factors and hormones and to neo-angiogenesis and neovasculogenesis during endochondral bone formation. In addition data on the expression of new genes active in differentiating chondrocytes such as Ex-FABP, CASP and KIAA0009 protein will be reported. Finally, information will be given on tissue engineering and cell therapy for cartilage and bone repair.

Induction of chondrocyte differentiation in culture

In the early stage of differentiation, chondrocyte phenotype is unstable. Culturing of early differentiation stage chondrocytes as cells adherent to a substratum induces "per se" dedifferentiation. Cells acquire a more elongated, fibroblastic morphology and switch from the synthesis of cartilage specific extracellular matrix macromolecules, such as aggrecan, type II, and other cartilage specific collagens, to the synthesis of macromolecules characteristic of prechondrogenic cells, such as type I collagen. Dedifferentiated cells expanded in culture maintain a chondrogenic potential and reacquire the chondrocyte phenotype when transferred into permissive culture conditions, such as a decreased cell substratum interaction. In the following two paragraphs, we will give information on the chick embryo chondrocyte culture system that we have developed starting from cells derived from early stage chick embryo tibiae.

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From prechondrogenic cells to hypertrophic chondrocytes

Chick embryo chondrocytes are dissociated from early stage embryo tibiae and plated as adherent cells in standard tissue culture Petri dishes. Under these conditions chondrocytes progressively dedifferentiate assuming a fibroblastic morphology and switch to the synthesis of type I collagen and other proteins characteristically expressed by prechondrogenic cells (Castagnola *et al.*, 1986). After reaching confluence, dedifferentiated chondrocytes can be trypsinized and passaged as such in adherent culture conditions. When passaged, dedifferentiated chondrocytes are transferred into dishes coated with agarose (suspension culture conditions); within a few hours they form small cell aggregates. During the first week in suspension culture, the aggregates increase in size and progressively start to release single isolated hypertrophic chondrocytes. After 2-3 weeks the culture is entirely made of single isolated hypertrophic chondrocytes. Soon after their transfer into suspension, cells resume the synthesis of type II and IX collagens and of other cartilage specific proteins. Later they also express high levels of the hypertrophic specific type X collagen. Interestingly, type VI collagen is the first collagen raising soon after the beginning of the suspension culture (Quarto *et al.*, 1993).

Standard culture medium does not contain ascorbic acid. In the presence of ascorbic acid, an important cofactor required for the hydroxylation of collagen molecules, hypertrophic chondrocytes are not released and small fragments of "*in vitro*" made cartilage are organized (Tacchetti *et al.*, 1987)

From hypertrophic chondrocytes to osteoblast-like cells

After enzymatic digestion of the extracellular matrix, single isolated hypertrophic chondrocytes are replated in the presence of ascorbic acid and β glycero-phosphate on plastic dishes (anchorage permissive substratum (Descalzi Cancedda *et al.*, 1992). Under these culture conditions hypertrophic chondrocytes acquire an elongated or star shaped morphology, resume cell proliferation, although at a low rate, progressively stop to deposit cartilage specific extracellular matrix and differentiate in osteoblast-like cells. Some alkaline phosphatase positive cells are observed and their number progressively increases. Eventually calcium mineral is deposited in the extracellular matrix. Deposition of calcium mineral is facilitated by high cell densities. At the same time, changes occur in the collagen types released by the cells. Between the first and the second week of culture the cells stop synthesizing cartilage specific type II and X collagens and switch to the synthesis of type I collagen. Mineralization occurs along fibrils of type I collagen. No proteoglycan "granules" are found in the cartilage matrix. Addition of retinoic acid, a differentiating agent, to the culture medium, accelerates further maturation of hypertrophic chondrocytes to osteoblast-like cells. The switch in the collagen production occurs as soon as 2 days later and extracellular matrix mineralization within 2 weeks.

Fate of hypertrophic chondrocytes

Further differentiation to osteoblast-like cells

It is generally believed that hypertrophic chondrocytes are terminally differentiated cells, whose ultimate fate is degeneration and death but the alternate view that growth plate hypertrophic chondrocytes transdifferentiate to osteoblasts has also been suggested (for a review see Cancedda *et al.*, 1995).

In a previous section of this review we have reported that when chick hypertrophic chondrocytes were transferred to substrate dependent culture conditions in the presence of ascorbic acid, cells showed a change in morphology, became more elongated and flattened, expressed alkaline phosphatase and eventually mineralized (Descalzi Cancedda *et al.*, 1992). We have also previously reported that when retinoic acid is added to the hypertrophic chondrocyte culture between day 1 and day 5, the maturation of the cells to the osteoblast-like stage was highly accelerated. We have defined this additional differentiation stage as an osteoblast-like stage.

The growth of hypertrophic chondrocytes undergoing this further differentiation process was compared to the growth of hypertrophic chondrocytes maintained in suspension culture as such (Gentili *et al.*, 1993). The proliferation rate was significantly higher in the adherent hypertrophic chondrocytes differentiating to osteoblast-like cells. Cells stopped proliferating when mineralization of the extracellular matrix occurred. The ultrastructural organization of the mineralized osteoblast-like cell cultures was investigated. Cells are embedded in a dense meshwork of type I collagen fibers and mineral is observed in the extracellular matrix associated with collagen fibrils.

Differentiation of hypertrophic chondrocytes toward an osteoblast-like phenotype occurs *in vivo* in the hypertrophic cartilage of chick embryo tibiae underneath early/prospective periosteum and in cartilage around vascular canals (Galotto *et al.*, 1994). Synthesis of type I collagen by hypertrophic chondrocytes was shown by immunolocalization of the C- propeptide. Evidence that hypertrophic chondrocytes may resume cell proliferation was derived from 5-bromo-2'deoxyuridine (BrdU) labeling experiments. BrdU labeled hypertrophic chondrocytes were located at the lateral edges of the hypertrophic cartilage, facing the periosteum, and around vascular canals. By enzyme cytochemistry it was shown that *in vivo* further differentiating hypertrophic chondrocytes express alkaline phosphatase, an indicator of initial mineral deposition.

Additional support to the concept that hypertrophic chondrocytes may further differentiate to osteoblast-like cells and participate to the initial bone formation "*in vivo*" was derived from studies performed with specific monoclonal antibodies raised against chick hypertrophic chondrocytes and osteoblasts (Galotto *et al.*, 1995).

Specific transcription factors certainly play a role in the process control. It is worth noting that, in mice, disruption of the zinc finger gene Krox-20 by homologous recombination prevents the last step of chondrocyte differentiation into osteoblast-like cells (Levi *et al.*, 1996). The mice develop skeletal deformities, including reduced length and thickness of long bones and severe reduction of calcified trabeculae. Periosteal bone formation does not appear to be affected in the homozygous mutant.

The transferrin loop

During the *in vitro* differentiation into osteoblast-like cells, hypertrophic chondrocytes transiently secrete large amounts of an 82 kDa glycoprotein (Gentili *et al.*, 1993). The protein has been purified from conditioned medium and identified as ovotransferrin. Ovotransferrin was transiently expressed also in cultures supplemented with retinoic acid. High levels of expression were observed in the early phase of the culture and when culture conditions

allowed extracellular matrix assembly, i.e. in the presence of ascorbic acid (Gentili *et al.*, 1994). Cells expressing ovotransferrin also coexpress ovotransferrin receptors. By immunoblot analysis, receptor proteins were detected at a very low level in extract from differentiating and hypertrophic chondrocytes and at a higher level in extract from hypertrophic chondrocytes undergoing differentiation to osteoblast-like cells and from mineralizing osteoblasts.

The expression of ovotransferrin "*in vivo*" during chick embryo tibia development was also investigated. By immunocytochemistry and "*in situ*" hybridization, ovotransferrin was initially detectable in the cartilaginous bone rudiments. At later stages, the protein was localized in the pre- articular region of the bone, in hypertrophic cartilage, in zones of cartilage erosion and in the osteoid at the chondro-bone junction. At the same time, chondrocytes at all stages of differentiation express a low level of the ovotransferrin specific receptor. High levels of the receptor were detectable in the 13 day old tibia in the diaphysis collar of stacked-osteoprogenitor cells and in the layer of derived osteoblasts.

Based on these results, the existence of autocrine and paracrine loops involving ovotransferrin and its receptor during chondrogenesis and endochondral bone formation was proposed (Gentili *et al.*, 1994).

Priming of bone formation

During bone growth and remodeling of growing and adult vertebrates, new formed bone is always deposited on a pre-existing mineralized bone, whereas the initial deposition of bone during embryogenesis occurs in a non-mineralized tissue. The cellular architecture of initial osteogenic sites was investigated by light, confocal and electron microscopy in bones which form by membranous ossification (not forming on a cartilage model), as fetal calvarial bones, and in the ossifying bony collar of endochondral bones (formed on a cartilage model) (Riminucci *et al.*, 1998). Bone sialoprotein (BSP), a protein expressed during early phases of bone deposition and controlling both mineral formation and bone cell-matrix interactions, was used as a marker of initial bone formation. At all sites, BSP was initially synthesized by cells located in a characteristic *vis à vis* pattern. In all cases, the first mineralizing matrix was observed in the BSP-immunoreactive extracellular matrix between the two cell rows. In bone formed by membranous ossification the two rows of cells are made by osteoblasts, whereas in perichondral osteogenesis, the *vis à vis* pattern comprises osteoblasts differentiating from the perichondrium/periosteum, and early hypertrophic chondrocytes located at the periphery of the cartilage rudiment. We have suggested that for the deposition of the initial bone structures (bone priming), the spatial organization (*vis à vis*) of cells competent to deposit a mineralizing matrix determines the polarized deposition of bone. In the case of endochondral bone formation, the priming of bone deposition involves and requires cells differentiating from early hypertrophic chondrocytes (Fig. 1).

In summary, our observations, made both "*in vitro*" and "*in vivo*", point to the fact that death is not necessarily the only fate of hypertrophic chondrocytes. We have proposed that all early hypertrophic chondrocytes have the inherent potential to differentiate to osteoblast-like cells and to contribute to the initial bone formation. "*In vitro*", changes in the culture conditions trigger the further differentiation to osteoblast-like cells. "*In vivo*", only chondrocytes positioned at the "borderland" between cartilage and non-cartilage

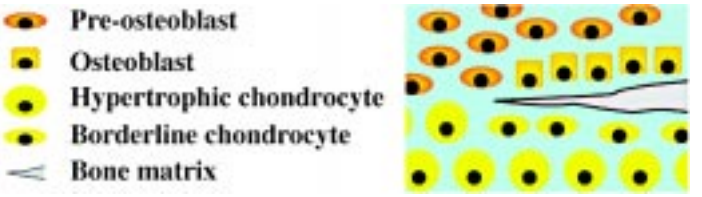

Fig. 1. Priming of bone formation in a developing long bone. *Borderline hypertrophic chondrocytes (osteoblast-like phenotype) and frank osteoblasts derived from undifferentiated perichondral cells participate in the "vis à vis" row of bone forming cells. Due to this peculiar cell organization, high concentrations of critical proteins are reached between the two cell layers, also in the presence of cells secreting proteins in a non-polarized manner. Local enrichment of critical matrix proteins, such as the bone sialoprotein BSP, favors mineral deposition and matrix-directed polarity of osteoblasts. In this way, the initial bone trabeculae are formed.*

osteogenic tissues, characterized by a peculiar microenvironment in terms of matrix-originating signals and endocrine/paracrine environment, undergo differentiation to bone producing cells (Bianco *et al.*, 1998). The name "borderline chondrocytes" indicate both the specific location of these hypertrophic chondrocytes and their dual differentiation potential. Hypertrophic chondrocytes located in different cartilage areas are exposed to an inappropriate microenvironment, cannot differentiate to osteoblast-like cells and undergo apoptosis.

Cartilage progenitor cells and the onset of chondrogenesis

Bone marrow stromal cells: progenitor or stem cells?

It is generally believed that chondrocyte progenitors are undifferentiated cells of mesenchymal origin present not only in the limb bud and other sites of initial bone morphogenesis, but also in the perichondrium/periosteum, in the bone marrow stromal compartment (Bone Marrow Stromal Cells, BMSC) and possibly elsewhere. In particular BMSC can differentiate to different lineages: osteoblasts, chondrocytes, adipocytes, myocytes. When implanted "*in vivo*", these cells can reconstitute bone tissue and cartilage under certain conditions. Conditions have been developed for BMSC isolation and expansion (Martin *et al.*, 1997). Bone marrow from healthy donors was used as cell origin; BMSC were selected on the basis of their capacity to adhere to the plastic of the culture dishes and to proliferate by forming fibroblastoid colonies, CFU-f.

It has been crucial to identify culture media allowing the selection and posterior expansion of BMSC, while maintaining their differentiation potentials unchanged. Several growth factors and hormones have been tested. We have concluded that FGF-2 was the most effective in promoting BMSC proliferation, maintaining cells in a more immature state and allowing "*in vitro*" expansion of human osteo- chondro- adipo-progenitors which, in the proper conditions, can differentiate "*in vivo*" and "*in vitro*" and form specific tissues.

The presence of Mesenchymal Stem Cells (MSC) in the stromal cell population has been proposed (Caplan, 1991). Mesenchymal stem cells (MSC) are defined as pluripotent cells dividing many times and self-maintaining throughout the organism life and whose progeny eventually gives rise to skeletal tissues: cartilage, bone, tendon, ligament and marrow stroma. By definition, such cells exist

during embryogenesis. It is still questionable whether these cells persist throughout adult life.

We have investigated stem features of human bone marrow stromal cells (BMSC) "*in vitro*" and tried to answer the following questions: 1) Do clones derived from a single BMSC display a multilineage differentiation potential?, 2) Are they limited to a defined number of mitotic divisions?, 3) Do they maintain a constant doubling time throughout the "*in vitro*" culture?, 4) Do they maintain their own multilineage differentiation potential after a number of mitotic divisions?, 5) Do BMSC express telomerase activity throughout mitotic divisions? and 6) Are they stem cells for the mesenchymal lineages or are they part of a plastic mesenchymal cell progenitor compartment?

The differentiation potential of 185 non-immortalized human bone marrow stromal cell clones has been investigated (Muraglia *et al.*, 2000). All clones, but one, differentiated into the osteogenic lineage. Most clones (>90%) displayed an osteo-chondrogenic potential. About 1/3 of the clones displayed the osteo-chondro-adipogenic potential. Clones with a differentiation potential limited to the osteo-adipo- or to the chondro-adipogenic phenotype, as well as pure chondrogenic and adipogenic clones were never observed. Life span was limited to about 22 cell doublings. Clones progressively lost the adipogenic and the chondrogenic differentiation potential at increasing cell doublings. Telomerase activity was not detected in bone marrow stromal cells (Banfi *et al.,* unpublished results).

Our data indicate that bone marrow stromal cells display a multilineage differentiation potential which progressively disappears during culture. They do not express telomerase activity and have a limited proliferation ability. Therefore they do not posses all the features of stem cells. Culture conditions may obviously be misleading. Moreover, whereas the best demonstration of the existence of a hemopoietic stem cell is provided by "*in vivo*" studies (the ability to fully reconstitute an organism myeloablated), the same evidence is not straightforward for the stromal compartment. In fact patients who have received bone marrow transplant display a normal reconstitution of the hemopoietic system, whereas the marrow stroma, which is substantially damaged by the myeloablative pre-transplant conditioning, does not show any sign of recovery either by recipient's or by donor's CFU-f (Galotto *et al.*, 1999).

Taken together these observations indicate that BMSC must be considered as early mesenchymal progenitors, and not as mesenchymal stem cells.

Cell-cell interactions and precartilage condensation

During embryogenesis onset of chondrogenesis is always preceded by precartilage condensation. Mesenchymal cell condensation in chick limb bud occurs at embryonic stage 22 and it is the starting event of chondrogenesis. A correlation between cell aggregation and formation of cartilage nodules has been similarly observed in culture. In micromass cultures of chick limb bud mesenchymal cells, extensive gap junctional communications are formed between differentiating chondrocytes; on the contrary, gap junctions are not observed in non chondrogenic cells differentiating into other connective tissues.

The molecular mechanisms activating chondrogenesis, following cell aggregation, are still unknown. Several mechanisms have been proposed. Apparently cell-cell contacts, mediated by membrane-bound cell adhesion molecules, play a major role. We have investigated the modulation of N-CAM and N-cadherin gene expression in the *in vitro* differentiating chick embryo chondrocyte culture (Tavella *et al.*, 1994). N-cadherin and N-CAM are developmentally regulated in differentiating chondrocytes. The timing of appearance of N-cadherin and N-CAM suggests that N-cadherin, mediating cell-cell contacts, initiates the "*in vitro*" cell condensation thereafter stabilized by N-CAM. The immunolocalization of these molecules in the cell aggregates revealed that, after transferring the cells into suspension culture, N-CAM and N-cadherin appeared first on the surface of all cells at the membrane regions participating in cell-cell contacts.

Modulation of chondrocyte differentiation by growth factors and hormones

Factors necessary to and controlling chondrogenesis and osteogenesis have been only partially identified. In order to study growth factors and hormones directly controlling chondrocyte proliferation and differentiation we have developed a chemically defined medium that would support complete maturation of growth plate chondrocytes to hypertrophy (Quarto *et al.*, 1992). When dedifferentiated cells are transferred into suspension culture in the presence of a serum free medium containing "physiological" concentration of T_3 (10^{-11}M), insulin (60 ng/ml), and dexamethasone (Dex) (10^{-9}-10^{-12} M), they revert to the chondrocyte phenotype and mature to type X collagen producing hypertrophic chondrocytes. The supplement of T_3 and insulin is mandatory to sustain proper chondrogenesis whereas Dex supports cell viability and modulates type X collagen expression. The same serum free medium containing the three compounds is unable to support cell growth, thus suggesting that additional factors may play a major role during chondrogenesis.

Several results indicate that a proper extracellular matrix assembly appears to be an absolute requirement for the expression of several soluble modulators by chondrocytes.

Retinoic acid-induced heparin binding factor (RIHB) belongs to a family of heparin binding and retinoic acid induced proteins transiently expressed during embryogenesis. We have investigated RIHB expression by avian embryo prechondrogenic cells and chondrocytes both "*in vivo*" and "*in vitro*" (Castagnola *et al.*, 1996). RIHB was detectable by immunofluorescence in the center of hind limb buds at embryonic stage 27 and in embryo tibia chondrocytes at stage 36. Both RIHB mRNA and protein were synthesized by cultured chondrocytes only when ascorbic acid was added to the medium (a condition promoting extracellular matrix assembly). Further addition of the iron chelating agent alpha-alpha' dipyridyl (a condition overcoming the ascorbic acid action and impairing extracellular matrix assembly) inhibited RIHB mRNA accumulation. In a previous section we have reported that also the expression of ovotransferrin by cultured chondrocytes was dependent upon the addition of ascorbic acid to the culture medium (Gentili *et al.*, 1993). The same is true in the case of secreted angiogenic activities (see following sections).

The effect of parathyroid hormone related protein (PTHrP) on late events in chondrocyte differentiation was investigated in the culture system (Zerega *et al.*, 1999). In developing bone rudiments, PTHrP, an autocrine/paracrine regulator of tissue development, regulates both the proliferation and the differentiation of

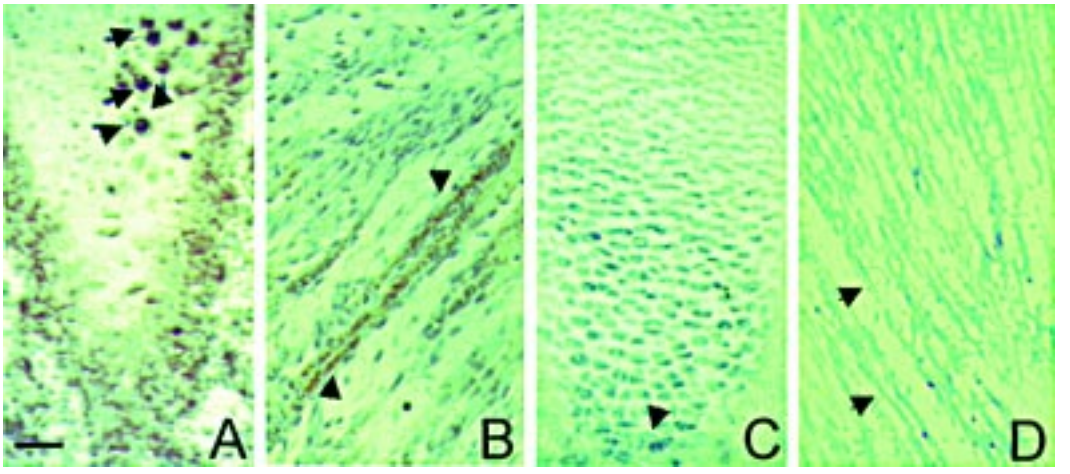

Fig. 2. Localization of Ex-FABP protein and mRNA in chick embryo (HH stage 36-37). *Panels A,C: sections of tibia. Panels B,D: sections of forming skeletal muscle.* **(A-B)** *Immunolocalization of the Ex-FABP protein.* **(C-D)** *Location of the Ex-FABP specific mRNA by in situ hybridization. Arrows point to positive hypertrophic chondrocytes in A) and C) and to positive forming muscle fibers in B) and D). Bar, 100 μm.*

chondrocytes by a negative feedback loop involving the soluble factor Indian Hedge-hog (Ihh) and its targets Patched (Ptc) and Gli (Vortkamp *et al.*, 1996).

In suspension culture (condition permissive for apoptosis) the formation of mineralized cartilage by hypertrophic chondrocytes follows the activation of type X collagen synthesis and is associated with the expression of alkaline phosphatase, arrest of cell growth, and apoptosis, as observed in growth plates *in vivo*. PTH/PTHrP repressed type X collagen synthesis, alkaline phosphatase expression and cartilage matrix mineralization. At the same time, cell proliferation was resumed and apoptosis blocked. In adherent culture conditions, where hypertrophic chondrocytes underwent further differentiation to osteoblast-like cells, PTHrP inhibited alkaline phosphatase expression and matrix mineralization while cell proliferation and type I collagen expression were not affected. Therefore PTHrP reverts and maintains cultured chondrocytes in differentiation stages earlier than hypertrophic chondrocytes (suspension), or earlier than mineralizing osteoblast-like cells (adhesion) thus preventing terminal differentiation and apoptosis.

Identification of new genes active in differentiating chondrocytes

Ex-FABP: a new lipocalin binding unsaturated long chain fatty acids and involved in endochondral bone formation

In our laboratory we have isolated and characterized a lipocalin binding fatty acids (Ex-FABP) developmentally regulated in chicken embryo. Ex-FABP is expressed during chondrogenesis and bone formation (Descalzi Cancedda *et al.*, 1988; Dozin *et al.*, 1992). *In vitro* Ex-FABP is highly expressed by differentiating hypertrophic chondrocytes (Descalzi Cancedda *et al.*, 1988). Interestingly, Ex-FABP is expressed also in the forming myotubes both "*in vivo*" and "*in vitro*" (Gentili *et al.*, 1998) (Fig. 2). The protein binds preferentially long-chain unsaturated fatty acids, such as oleic, linoleic and arachidonic acid (Descalzi Cancedda *et al.*, 1996).

Possible mammalian counterpart of the EX-FABP is the NRL/N-GAL protein (Zerega *et al.*, 2000). NRL (neu-related lipocalin) is a protein overexpressed in rat mammary cancer induced by activated neu (HER-2/C-erbB2) (Stoesz and Gould, 1995). NRL homologous in mouse is SIP24, an acute phase protein induced in the animal by turpentine injection (Liu and Nilsen-Hamilton, 1995). The human homologous N-GAL (Neutrophil Gelatinase Associated Lipocalin) is expressed in granulocytes (Kjeldsen *et al.*, 1994) and epithelial cells in inflammation and malignancy (Nielsen *et al.*, 1996). Ex-FABP expression is dramatically enhanced in hypertrophic chondrocytes and in *in vitro* formed myotubes by inflammatory stimuli and inhibited by non steroidal anti-inflammatory agents (Cermelli S. *et al.*, 2000).

We have proposed that Ex-FABP is a stress protein expressed in tissues where active remodeling is taking place during development and in tissues characterized by an acute phase response due to pathological conditions. We have also proposed that responses, characteristic of a local inflammatory status, are physiologically activated during endochondral bone formation.

Additional information on Ex-FABP and on the physiological inflammatory response is reported in a recent review article (Descalzi Cancedda *et al.*, 2000).

CASP (Cartilage ASsociated Protein) and the KIAA0009 protein

CASP (Cartilage ASsociated Protein) (Castagnola *et al.*, 1997; Tonachini *et al.*, 1999) and KIAA0009 protein (Tonachini *et al.*, unpublished results) are examples of new proteins expressed and synthesized by differentiating chondrocytes and identified in our culture systems. The identification of the genes coding for these two proteins has been accomplished by a subtraction cDNA cloning approach. A cDNA library enriched for cDNA specific for transcripts of hypertrophic chondrocytes was generated. The resulting cDNA clones were first screened to eliminate type X collagen specific cDNAs and then randomly selected and sequenced. The amino acid sequence was derived from the nucleotide sequence, specific oligopeptides were synthesized and antibodies produced.

During chick embryo development, CASP is first detected in the extracellular matrix of the area of condensing mesenchyme, where cartilage formation occurs, and later it is maximal in the matrix surrounding a subset of chondrocytes. Although CASP gene expression is not limited to cartilage, in other chick embryo tissues synthesis of the encoded protein is at a much lower level, occurs later and it is observed within the extracellular matrix of very restricted areas. For such reasons the protein has been named CASP (Cartilage ASsociated Protein). The restricted expression of CASP to a subset of chondrocytes is most evident in the developing

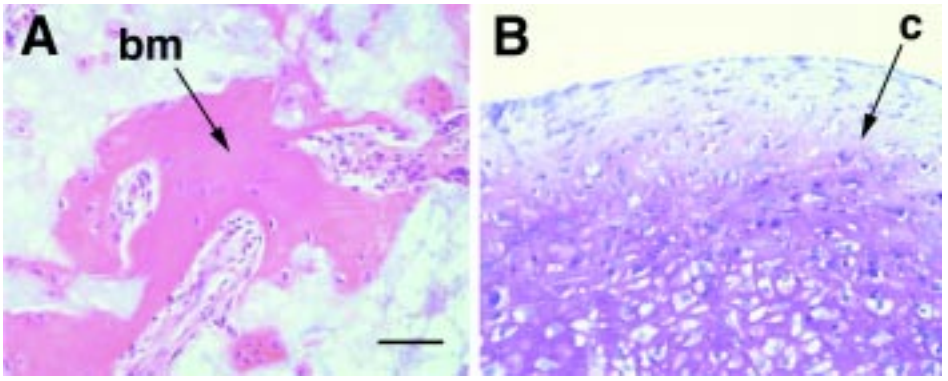

Fig. 3. Bone and cartilage formation by human BMSC. *The "in vivo" osteogenic and "in vitro" chondrogenic potential of human BMSC can be assayed by means of specific assays.* **(A)** *BMSC implanted "in vivo" in combination with porous bioceramics differentiate and deposit bone matrix (bm).* **(B)** *BMSC can be stimulated towards chondrogenesis "in vitro" as "pellet culture". In these conditions they form cartilagine (c). Bar, 50 μm.*

long bones. Matrix surrounding chondrocytes in the epiphysis extremities and in the lower hypertrophic zone is stained by CASP specific antibodies, whereas matrix surrounding cells located in the upper hypertrophic zone and in the proliferating zone is not (Castagnola *et al.*, 1997). We have also cloned and characterized the mouse and the human cDNA encoding for CASP and determined the chromosomal localization of the CASP genes that resulted to be on 9F3-F4 and 3p22 respectively (Morello *et al.*, 1999; Tonachini *et al.*, 1999).

With regard to KIAA0009 protein, the cDNA clone derived from the same library has been characterized very recently. Although the human cDNA counterpart was already cloned and sequenced (Nomura *et al.*, 1994) characterization of neither the gene expression pattern nor the encoded protein was made. As in the case of CASP, this protein localized in the cytoplasm is a novel protein that, although not strictly cartilage-specific, shows preeminent cartilage localization. During "*in vitro*" chondrocyte differentiation, KIAA0009 mRNA accumulation follows the maturation of cultured chondrocytes. RNA synthesis is enhanced by PTH (1-34) treatment of the cells. The peak accumulation of KIAA0009 mRNA occurs just before overt hypertrophy (Tonachini *et al.,* unpublished results)**.**

Neoangiogenesis and neovasculogenesis during endochondral bone formation

"*In vitro*" differentiating endochondral chondrocytes release angiogenesis inhibitors and stimulators into the culture medium. The balance between these two groups of factors varies during the process (Descalzi Cancedda *et al.*, 1995). Conditioned medium from cultures of dedifferentiated cells undergoing maturation to hypertrophic chondrocytes in suspension progressively inhibited both random migration and matrix invasion by endothelial cells. Conditioned medium from hypertrophic chondrocytes displayed the major inhibitory effect. The same medium prevented formation of tube-like structures by endothelial cells plated on basement membrane matrix gel. When the culture was performed in the presence of ascorbic acid, a condition leading to the formation of a mineralized tissue similar to calcified cartilage (see above), prevalence of angiogenic activity was detected by both an "*in vitro*" and an "*in vivo*" assay. The medium conditioned by osteoblast-like cells behaved in a similar way. Therefore, interactions of chondrocytes with their extracellular matrix are an absolute requirement for the expression of angiogenic activities by late hypertrophic chondrocytes.

Transferrin (Carlevaro *et al.*, 1997) and VEGF (Vascular Endothelial Growth Factor) (Carlevaro *et al.*, 2000) have been identified as major angiogenic factors in the chondrocyte conditioned medium and probably they play also a major role in cartilage neovascularization.

Affinity purified antibodies directed against transferrin completely inhibited the chemotactic activity exerted by the transferrins purified from different sources and species, including transferrin purified from chick hypertrophic chondrocytes and strongly, but not completely, inhibited the chemotactic activity exerted by the conditioned medium. By immunoblot analysis, the presence of transferrin receptors has been shown on the membranes of endothelial cells. Blocking antibodies against the transferrin receptor, added to the medium during the "*in vitro*" endothelial cell migration assay, lowered the chemotactic response to about 50% in comparison to control. Transferrin showed a remarkable angiogenic activity also in an "*in vivo*" assay (Carlevaro *et al.*, 1997). Similarly, antibodies against VEGF and antibodies directed against the VEGF receptor 2/Flk1 (VEGFR2) lowered to about 50% endothelial cell migration induced by chondrocyte conditioned media.

Soon before vascular invasion, in avian and mammalian embryo long bones, transferrin and VEGF were distinctly localized in growth plate hypertrophic chondrocytes, but were not observed in quiescent and proliferating chondrocytes.

Interestingly, when ovotransferrin receptor localization was investigated, it was observed that chondrocytes, at all stages of differentiation, express a low level of the specific receptor, whereas high levels of the receptor were present in the stacked-osteoprogenitor cells and in the layer of derived osteoblasts observed in the bone diaphysis (Gentili *et al.*, 1994). VEGF Receptor 2 colocalized with the factor both in hypertrophic cartilage "*in vivo*" and hypertrophic cartilage engineered "*in vitro*" (Carlevaro *et al.*, 2000). These findings suggest the existence of paracrine and autocrine loop in hypertrophic cartilage at the time of vascular invasion. It is to note that regardless of cell exposure to exogenous VEGF, VEGFR-2 phosphorylation was recognized in cultured hypertrophic chondrocytes, supporting the idea of an autocrine loop (Carlevaro *et al.*, 2000).

Tissue engineering and cell therapy for cartilage and bone repair

Recent advances in biotechnology and biomaterial science are converging to the new discipline of tissue engineering. Tissue

engineering can be defined as the science of fabricating new tissues for replacement and total regeneration. Cells and extracellular matrix are main ingredients for the successful tissue engineering. Biomaterials mimic the extracellular matrix and play a crucial role in supporting cell growth and differentiation and in delivering growth factors and other morphogenetic molecules. A considerable amount of work is in progress in our laboratory in the area of composite biomaterials, obtained by the associations of cells with resorbable scaffolds, and particularly of biomaterials suitable for cartilage and bone repair. The applicability of new biomaterials to cartilage and bone repair can be investigated taking advantage of the experimental systems we have developed (Fig. 3).

Cartilage is among the most frequently transplanted tissues. Several pathological conditions of articular cartilage (repair of inflammatory, congenital and traumatic lesions) require grafts of new tissue to achieve a complete functional recovery. Such a requirement results from the inability of articular cartilage to spontaneously repair, thus being a tissue that is neither vascularized nor innervated. The tentative to rebuild tissues from autologous or allogeneic cells expanded in culture "ex vivo" has become a growing field in the world of biomedical sciences. The cellular sources for cartilage repair can be differentiated chondrocytes and/or chondrocyte progenitors as bone marrow stromal cells (BMSC). Both cell types can be harvested from the patient and expanded "*in vitro*" until they reach a cell number suitable for lesion repair. A full characterization of the cultured cells with particular focus on their proliferation and differentiation potential is mandatory before cells can be considered for transplantation.

Large bone defect repair has always represented a problem of difficult resolution both from a surgical and biological point of view. Marrow-derived osteogenic precursor cells (BMSC) combined with hydroxyapatite (HA) potentially represent a significant advancement in skeletal reconstruction. We have shown the validity of this model for the repair of a critical size tibial defect in sheep, where segmental healing has been successfully achieved by BMSC-HA implants (Kon *et al.*, 2000). A pilot clinical study with human patients is currently underway

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