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Hsp and chaperone distribution during endochondral bone development in mouse embryo

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Abstract The process of endochondral bone formation was examined with regard to expression of seven heat shock proteins (Hsps): two small Hsps, the constitutive and the inducible forms of the 70 and the 90 Hsp families, the collagen chaperone Hsp47, and a cytosolic chaperone, TCP-1α, using immunohistochemistry. Around day 15.5 of embryogenesis the calcification of the long endochondral bones occurs through progressive replacement of the cartilaginous scaffold (rich in type II collagen) with an ossified matrix (rich in type I collagen), and thus a longitudinal section of limb bone recapitulates all the steps of chondrogenesis and the early steps of osteogenesis. We observed that all these Hsps and chaperones are differentially expressed during bone development in a stage-specific pattern reaching very high levels at some specific stages. The involvement of chaperones during these important differentiation steps will be discussed.

INTRODUCTION

Development of long bones occurs by the process of endochondral ossification. It begins with the condensation of mesenchymal cells in the developing limb bud which then differentiate into proliferating chondrocytes (the initial cartilage elements). Cells around the cartilage primordium flatten and form the dense perichondrium which separate chondrocytes from surrounding tissues. At a later stage, chondrocytes in the center of the cartilage undergo further differentiation into hypertrophic chondrocytes (later cartilage elements) which is a prerequisite for the next steps of ossification. At the same time mesenchymal cells in the perichondrium differentiate into osteoblasts. Thus two types of bone arise from endochondral ossification: 1) the periosteal bone where perichondrial osteoblasts deposit the bone matrix in a cylinder around the diaphyse of the bone (Osdoby and Caplan 1981); 2) the endosteal bone where the osteoprogenitor cells colonize and ossify a pre-existing cartilage scaffold. Endosteal bone formation coordinates the formation of cartilage tissue and the subsequent replacement of the cartilage model by bone. It involves a series of events including formation of the cartilage, its hypertrophy and calcification, vascular invasion, appearance of osteoblasts and formation of the bone (for review see Cancedda et al. 1995; Erlebacher et al. 1995). The development and growth of the skeletal tissues is characterized by profound changes in the expression of extracellular-matrix genes. Collagen is the major macromolecular component of the extracellular matrix of the endochondral bone and it plays an important role in determining size, shape and strength of these tissues. Bone and cartilage consist of genetically distinct collagens: among the most important species, type I collagen is specific to osteoblasts (bone) and type II collagen is specific to chondrocytes (cartilage). Therefore, endochondral ossification requires the gradual replacement of matrix collagen of type II with bone type I collagen (von der Mark and von der Mark 1977; Yasui et al. 1984; Alini et al. 1992), followed by the deposition of calcium onto the matrix by the chondrocytes. It has been shown that during the mineralization of the extracellular matrix the hypertrophic chondrocytes switch from aerobic to anaerobic respiration (Brighton and Hunt 1974). This oxidative

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Table 1 References of the primary antibodies used in the following analysis

Primary antibody	Purchaser	Reference	Origin	Dilution
Hsp25	StressGen Biotechnologies	SPA801	Rabbit (polyclonal)	1/100°
α-Bcrystallin	Novocastra	NCL6ABcrys	Rabbit (polyclonal)	1/100°
Hsp47	StressGen Biotechnologies	SPA410	Mouse (monoclonal)	1/100°
Hsc70	StressGen Biotechnologies	SPA815	Rat (monoclonal)	1/100°
Hsp70	Amersham	anti-Hsp72	Mouse (monoclonal)	1/300°
Hsp90α	Affinity Bioreagent Neshanic Station, NJ	PA3–013	Rabbit (polyclonal)	1/40°
Hsp90β	Affinity Bioreagent Neshanic Station, NJ	PA3–012	Rabbit (polyclonal)	1/40°
TCP-1α	R.Melki's gift		Rabbit (polyclonal)	1/150°
Type I Collagen	Rockland, Gilberville, PA	anti-collagen type I	Rabbit (polyclonal)	1/100°
Type II Collagen	Rockland, Gilberville, PA	anti-collagen type II	Rabbit (polyclonal)	1/100°

metabolism change causes a decrease in cellular ATP and a shift to a phosphocreatine mediated energy pathway (Shapiro et al. 1992). These metabolic changes result in the deposition of calcium from the mitochondria (Brighton and Hunt 1974).

We investigate here, in the absence of stress, the distribution of six major (cognate and/or inducible) heat shock proteins (Hsps): Hsp90α and β, Hsp70, Hsc70 and the sHsps, Hsp25 and α-Bcrystallin during endochondral bone formation. We compare their expression pattern with those of two specialized chaperones: the cytosolic chaperone TCP-1α, involved in actin and tubulin folding (Melki and Cowan 1994), and the endoplasmic-reticulum-resident heat shock protein Hsp47. Hsp47 plays a major role as a molecular chaperone essential for the folding and oligomerization of collagens (Saga et al. 1987; Nagata 1996). Our observations show that the different Hsps are expressed in a dynamic spatio-temporal pattern along the endochondral bone, reaching in some cells very high levels. The cell and developmental specificity of expression of each Hsp argues for cell-specific functions.

MATERIALS AND METHODS

Animals

Embryos from outbred OF1 mice (Iffa Credo) were taken at E15.5. The mid-point of the dark interval during which the mating occured was designated as day 0 and the embryo was considered to be at E0.5 on the morning following breeding. The females were killed by cervical dislocation and the embryos were dissected free from the uterus in phosphate buffered saline (PBS). At least three embryos were examined and the same pattern of localization was reproducibly observed.

Immunostaining on cryosections of forelimbs

Embryos were fixed overnight by immersion in 4% paraformaldehyde in phosphate buffer (0.12M, pH7.2) at 4°C. Forelimbs including scapula were isolated. Samples were embedded in 7.5% gelatin/15% saccharose in PBS, frozen at −56°C (using isopentane immersed in liquid nitrogen), and cryosectioned (17 μm sections). Sections were stored at −20°C for at most 1 month in a dessicated box.

Sections were rehydrated in 50 mM NH_4Cl/PBS, then submerged in a blocking solution (3% BSA in PBS), containing 0.5% TritonX-100 to permeabilize, and 0.6% hydrogen peroxide as susbtrate to block the endogenous peroxidase activity of the tissues. After rinsing, serial sections were collected and alternatively incubated with one of the antibodies raised against different Hsps and chaperones, or against type I or type II collagen (Table 1).

Sections were incubated for 1 h, at room temperature, with one primary antibody, at the convenient dilution in the blocking solution enriched in 0.1% TritonX-100, rinsed, then incubated for 30 min with the appropriate horseradish-peroxidase conjugated secondary antibody (Table 2). Peroxidase activity was revealed using 0.03% diaminobenzidine tetrahydrochloride (Sigma)/0.005% H_2O_2 in 0.1 M Tris-HCl (pH 7.6). The reaction was stopped in distilled water and the sections mounted in Mowiol.

Table 2 References of the secondary antibodies used in the present work

PO-conjugated-antibody	Purchaser	Reference	Origin	Dilution
Anti-rabbit IgG	Promega	W4011	Goat	1/100°
Anti-rat IgG	Boehringer Mannheim	1 348 752	Sheep	1/200°
Anti-mouse IgG	Sigma	A4416	Goat	1/100°

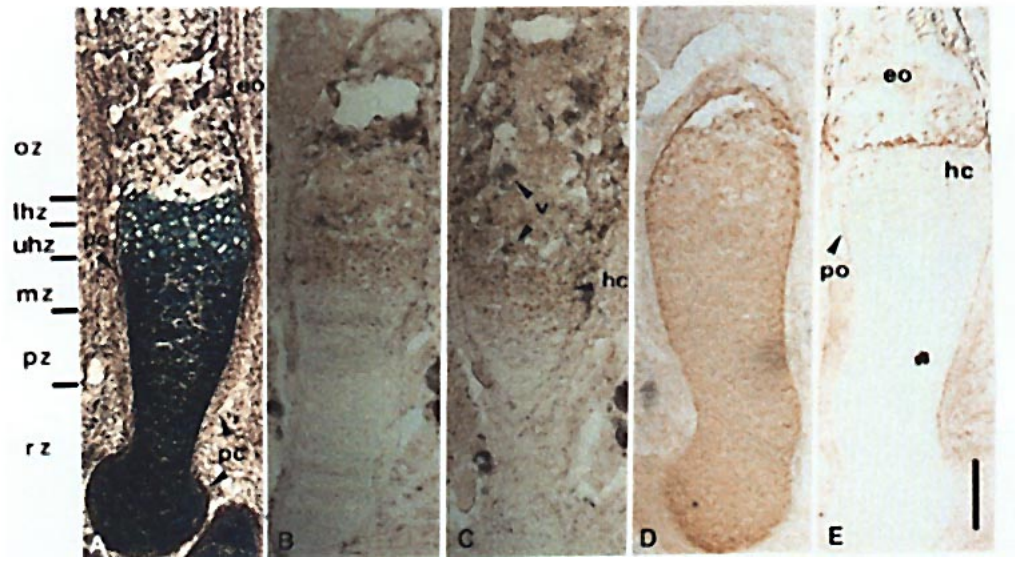

Fig. 1 Longitudinal sections of a day 15.5 (E15.5) mouse embryo humerus. (A) Alcian blue/haematoxylin stained section. Alcian blue strongly stains the cartilaginous matrix throughout the different steps of chondrogenesis: resting zone (*rz*), proliferating zone (*pz*), maturing zone (*mz*), upper hypertrophic zone (*uhz*), lower hypertrophic zone (*lhz*), ossification zone (*oz*). (B–E) Immunolocalization of Hsc70 (B), TCP-1α (C), compared to type II collagen (D), and type I collagen (E). Endosteal bone (*eo*), hypertrophic chondrocytes (*hc*), perichondrium (*pc*), periosteal bone (*po*), vessel (*v*). Bar 200μm.

Staining methods

Parallel sections were stained to allow visualization of the different steps of endochondral bone formation: either with alcian blue, counterstained with haematoxylin to stain the cartilage, by von Kossa's method to demonstrate calcium deposits, or with an histochemical reaction (Boehringer kit) specific for the alkaline phosphatase activity which accompanies the mineral deposit.

RESULTS

Description of the experimental approach

Endochondral ossification occurs via an orderly sequence of steps of chondrocyte differentiation beginning with cell proliferation and ending with chondrocyte hypertrophy and death or further maturation in osteoblast-like cells (Cancedda et al. 1995). Whereas at day 15 of mouse embryogenesis, long bones are only cartilaginous cell condensations, at day 15.5 in the upper limb a considerable degree of periosteal and endosteal ossification is seen in the middle third of the scapula, the humerus, the ulna and the radius whereas the proximal and distal parts of the bone remain un-ossified (Fig. 1) (Kaufmann 1992).

Therefore, we decided to focus our studies at day 15.5. This stage of development is particularly amenable to the study of endochondral ossification: a longitudinal section of bone recapitulates all the steps of chondrogenesis in the distal two-thirds of the bone, whereas the transition between cartilage and bone at initial steps of ossification are clearly detectable in the medial third of the bone. All these steps will later be concentrated in the narrow area of the growth plate, localized in the epiphyse of long bones during further development. At E15.5, despite the proximo-distal progression of differentiation, scapula, humerus, radius and ulna display a similar bone developmental stage, although the scapula anatomy allows a better analysis of the chondro-osseous junction.

The successive steps in chondrogenesis and osteogenesis were characterized on parallel sections by specific stainings: alcian blue for cartilage, alkaline phosphatase detection for the mineralisation step or von Kossa reaction for bone. Changes in the molecular composition of the extracellular matrix were followed by immunolocalization of two major collagens: type II collagen, which is a marker of the cartilaginous matrix, and type I collagen which is specific to the bone matrix.

Antibodies were chosen according to their ability to recognize only one member of each Hsp family. The specificity was demonstrated by Western blotting using in vitro differentiating chondrocyte and osteoblast extracts (Favet, unpublished results). Moreover, the specificity was further confirmed by the different pattern of expression which was observed (see later).

Hsp and TCP-1α distribution during chondrogenesis

Weak labeling with antibodies against Hsps and TCP-1a in perichondrium

At this stage, layers of fibroblast-like cells have formed around the cartilage, creating a boundary between the developing cartilage and the surrounding tissue. This

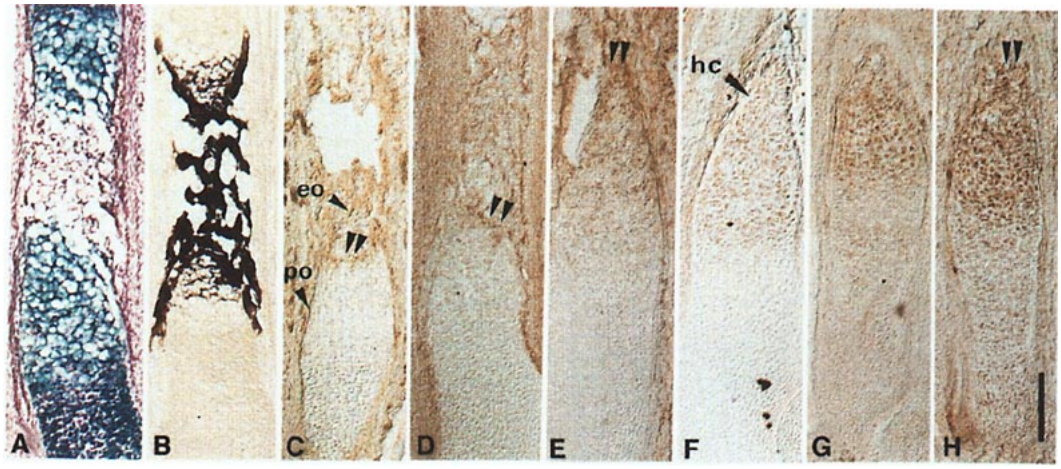

Fig. 2 Distribution of different Hsps (C–H) along a radius longitudinal section of a E15.5 mouse embryo, compared to alcian blue (A) and von Kossa (B) staining. Alcian blue reveals the cartilaginous matrix and von Kossa the endosteal bone of the middle third of the radius, the periosteal bone and the mineralizing matrix of hypertrophic chondrocytes. Hsc70 (C) and Hsp47 (D) localize at the ossification-front (double arrow heads), in the periosteum (*po*) and in the endosteal (*eo*) bone. Hsp25 (E), α-Bcrystallin (F) are strongly expressed in hypertrophic chondrocyte layers (*hc*). Hsp90 α (G) and β (H) are present all along the chondrogenesis. Bar 200μm.

perichondrium stains only faintly with anti-Hsc70, -Hsp90, -Hsp47 antibodies and more strongly with anti-Hsp25 in the articular primordiae (not shown).

Very specific expression of Hsps in the chondrocyte lineage
Beginning with the epiphyseal plates at each end of the growing bone, we will move progressively towards the middle of the bone, and describe Hsp and chaperone expression in:

1. the reserve zone (rz)
2. the proliferative zone (pz)
3. the maturing zone (mz)
4. the upper hypertrophic zone (uhz)
5. the lower hypertrophic zone (lhz) where calcification occurs (Fig. 1) (Alini et al 1992; Cancedda et al 1995; Erlebacher et al 1995).

The reserve zone At the sub-ridge level of the bone tips, under the perichondrium, tightly packed amalgams of spherical cells show strikingly different patterns of localization for the different Hsps. The two Hsp90s, α and β, are present in these non-dividing cells, the level of Hsp90β being higher. Hsp47, TCP-1α and Hsc70 are faintly expressed, and the two small Hsps only at a very low level. Hsp70 is clearly absent (not shown). As the ossification front extends further outwards, this remaining cartilage will form the growth area of the bone. In agreement with the observations of von der Mark and von der Mark (1977), we found type II collagen in this early cartilaginous structure. This synthesis occurs in the presence of only a low amount of Hsp47. In opposition to immunocytological data in chicken (von der Mark 1980), we did not observe, with our antibody, type I collagen synthesis in these pre-chondrogenetic limb mesenchymal cells.

The proliferative zone In this region where dividing cells give rise to columns of flattened chondrocytes (Figs 1 and 2), Hsps and TCP-1α distribution are very similar to that in the reserve zone, except that the expression level of Hsp90 is lowered (Fig. 2 G,H). The cartilaginous matrix is known to be mainly composed of type II, IX, XI collagens and proteoglycans. Type II collagen is abundant in the proliferative zone (Fig. 1D). Besides the collagens, the proteoglycan chondritine and keratan sulfates confer the characteristic alcian blue stainability (Figs 1A and 2A) (Hardingham and Fosang 1992).

The maturing zone The main feature of this level where the chondrocytes begin to enlarge into hypertrophic chondrocytes, is a slight enrichment in Hsp and chaperone synthesis (Figs 1 and 2). The small Hsps are clearly expressed here. Hsp70 is still absent. Type II collagen remains the main component of the extracellular matrix.

The upper hypertrophic zone This region is characterized by cells enlarged 5- to 10-fold. The cells reach their maximal size; cell enlargement is accompanied by a reduction of matrix volume and consequently of type II collagen. The upper hypertrophic chondrocytes are characterized by a strong alkaline phosphatase activity (Fig. 3): in cartilage calcification, intracellular calcium is released through matrix vesicles of the chondrocyte membrane which adhere to the molecules of the extracellular matrix and these vesicles were shown to be rich

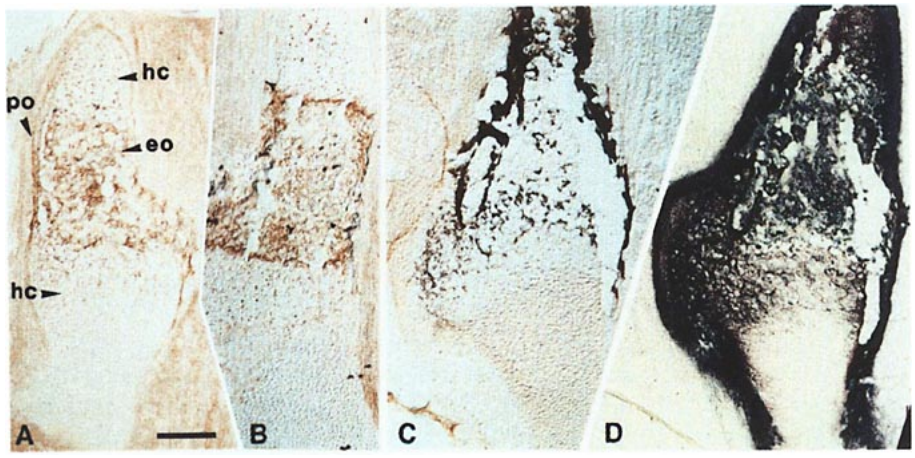

Fig. 3 Distribution of Hsp47 (A) compared to type I collagen (B), von Kossa staining (C) and alkaline phosphatase activity (D) across a longitudinal section of a E15.5 embryo scapula. Hsp47 co-localizes mainly with these three bone matrix markers. Endosteal bone (*eo*), hypertrophic chondrocytes (*hc*), periosteal bone (*po*). Bar 200μm.

in alkaline phosphatase (Brighton and Hunt 1974; Osdoby and Caplan 1981). Hsp and chaperone synthesis increases dramatically: substantial amounts of Hsp90, small Hsps, Hsc70, and TCP-1α are present in the cytoplasm of the cells. Hsp90α is synthesized at a level similar to the level of Hsp90β. The detectable Hsp47 enrichment is relatively meagre compared to the other Hsps (Fig. 2D). Despite the fact that small Hsps and the Hsp90 synthesis is obviously increased at this step, the synthesis of inducible Hsp70 is not detectable.

The lower hypertrophic zone A new extracellular matrix component, the type I collagen, is substituted to type II collagen. This coats the surface of the cartilage and the lacunae of the hypertrophic chondrocytes and forms the osteoid seam (Fig. 1) (von der Mark and von der Mark 1977), leading to the calcification of the lowermost part of hypertophic cartilage (Fig. 1E). The intracellular labeling of the upstream hypertrophic chondrocytes with anti-collagen I is indicative of the biosynthesis of the collagen before its secretion and incorporation to the bone matrix. At this stage all the Hsps, including Hsp70, are now present. The content of Hsp90, Hsc70, Hsp25, α-Bcrystallin and TCP-1α increases according to a gradient from the maturating zone to the hypertrophic zone; the highest level being reached in the lower hypertrophic zone (Figs 1 and 2). In contrast, Hsp70 demarcates exactly the type I collagen domain which corresponds to the lowermost part of the hypertrophic zone located in the mid-diaphyseal region (Fig. 2C). Hsp47, which was weakly expressed in the four earlier zones characterized by a type II collagen cartilaginous matrix, is also more abundantly localized in regions of type I collagen production (Figs 2D and 3A).

HSP AND CHAPERONE DISTRIBUTION DURING OSTEOGENIC OSSIFICATION

Two populations of osteoblasts exist: (1) the initial osteoblast population of the perichondrium produces the periosteum and (2) vascular invasion of the cartilage scaffold transports both osteoblast and osteoclast progenitor cells which are involved in further bone development and remodeling (Caplan 1989). In addition it has been suggested that hypertrophic chondrocytes could transdifferentiate to osteoblasts (Thesingh and Scherft 1986).

Periosteal ossification

In the mid-diaphyseal region adjacent to the cartilage, the area of cartilage hypertrophy and the zone of periosteal mineralization are closely associated. High alkaline phosphatase activity in the presumptive periosteum indicates an osteogenic process (Fig. 3D). A specific cell population of the stacked-cell layer exhibits this activity associated with their plasma membrane before its appearance in the hypertrophic chondrocytes, independently therefore from vascular invasion (Osdoby and Caplan 1981). Type I collagen can be found in the periosteum (Fig. 3B) (von der Mark et al 1976). Hsp70, Hsp90β and Hsp25 are abundant in these osteoid matrix secretory osteoblasts (Fig. 4). TCP-1α and Hsc70 are present to a lesser extent.

Endosteal ossification

At E15.5, only the early developmental events of osteoblast differentiation are present. There is an invasion of vasculature through the outer limit with bony crust initiating resorption of the hypertrophic cartilage. Appearing with the vascular invasion are osteogenic cells

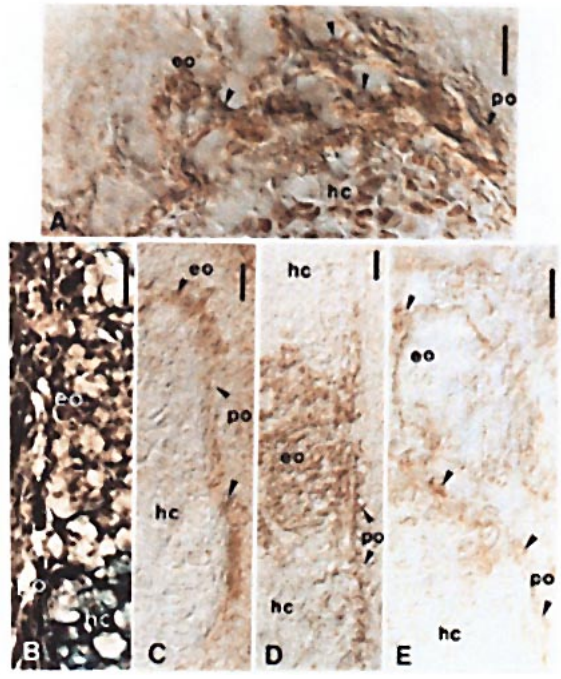

Fig. 4 E15.5 humerus longitudinal sections displaying, at higher magnification, localization of Hsp90β (A), Hsp70 (C), Hsp25 (D) and Hsp47 (E) in periosteal (po) or endosteal (eo) osteoblasts. Arrowheads point the differentiating osteoblasts. (B) Alcian blue/haematoxylin staining. Bar 50μ.

that produce type I collagen-rich matrix (osteoid) that further mineralizes. Bone marrow cavity, trabecular formation and osteoclast activity are later developmental events (Caplan 1989). As can be seen in Figure 4, several Hsps and chaperones are expressed in osteoblasts during either the proliferation or the differentiation phase. As in periosteum, Hsp70, Hsp90β, Hsp25 and Hsp47 are the most abundant (Fig. 4). Hsc70, TCP-1α, α-Bcrystallin and Hsp90α are also expressed, but at a lower level.

DISCUSSION

Our results show a very specific, non-coordinated and in most cases high, expression of Hsps during bone formation. Since Hsps are weakly expressed in the proliferating zone, the Hsp expression seems more related to the differentiating than to the proliferation steps. We observe a high level of Hsp expression in hypertrophic chondrocytes: the explanation for this is probably different for each of the different families of Hsps and we can only suggest some possible functions for these Hsps.

Hsp47, the collagen chaperone is expressed mainly during ossification

Hsp47, an endoplasmic-reticulum-resident Hsp, has been considered as a primary molecular chaperone for chains of procollagen (Natsume et al. 1994; Sauk et al. 1994). Collagens constitute the major macromolecular component of the extracellular matrix of the connective tissue. They begin their assembly within the lumen of the endoplasmic reticulum and are eventually secreted. Bone and cartilage consist of genetically distinct collagens: type I and type II respectively, which are the specific products of different cell types: osteoblasts or osteoblast-like cells of the lower hypertrophic zone for type I collagen, and chondrocytes for type II collagen. Our observation, that Hsp47 is more abundantly localized in regions of type I collagen production is in good agreement with those of Shroff et al. (1993) who reported that Hsp47 co-localized with type I collagen in murine bone and teeth. However, it is difficult to correlate our observations to those performed in other species: in zebrafish, the expression of the *hsp47* gene is mainly co-incident with the expression of type II collagen gene (*col2a1*) (Lele and Krone 1997). Despite that Hsp47 is able, in vitro, to bind to type I to type V collagens (Nagata 1996), Hsp47 interaction seems limited, in vivo, to one type of collagen (see earlier). Further experiments would be necessary to state precisely this in vivo specificity.

All Hsps are highly expressed in the chondrocyte transdifferentiation zone (lower hypertrophic zone)

Two hypotheses can be proposed for the high level of expression: either it is correlated with the transdifferentiation process or with the metabolic changes which occur in this tissue.

Hypertrophic chondrocytes may transdifferentiate into osteoblast-like cells: Endochondral bone formation is due to a sequence of events including hypertrophic cartilage formation, its invasion by blood vessels, the erosion of the cartilage and its replacement by bone. There are two different opinions of hypertrophic chondrocyte fate in the osteoid transition of the growth plate: that they degenerate and die, or that they transdifferentiate into osteoblast-like cells (Thesingh and Scherft 1986). In vitro and in vivo analyses in chicken by Galotto et al. (1994) demonstrated, using a chondrocyte marker, the simultaneous completion of the developmental program leading to the acquisition of osteogenic competence in both chondrocytes and committed osteogenic cells. Roach et al. (1995) showed that, in cultured femur cells from chicken, osteogenic differentiation of hypertrophic chondrocytes involves an asymmetric cell division: one cell remains viable and becomes osteogenic while the other is committed to die by apoptosis. A high level of p53 concomitant with cell death has recently been described in these cells (Trichilis and Wroblewski 1997). That Hsp25 and Hsp70 (Mehlen et al. 1996; Anderson 1997; Vanmuylder et al. 1997) might be involved in the balance

between differentiation and apoptosis (Arrigo et al. 1998) should not be neglected in this process.

Chondrocytes undergo a series of profound changes in energy metabolism in parallel with their morphological and functional modifications. In resting and proliferating zones, the cells generate energy by the mitochondrial and glycolytic pathways. In contrast, in hypertrophic cartilage, mitochondrial activity is limited and cells abruply shift to non-oxidative metabolism (Shapiro et al. 1992). Changes in nutrient sources and use of metabolic pathways have been shown previously to alter Hsp expression (Lanks 1986). However, the effect is not general. Despite the fact that small Hsps and Hsp90 synthesis is obviously increased at this step, the synthesis of inducible Hsp70 is not.

Hsp90 and small Hsp over-expression might be linked with the role of these proteins in signal transduction

Hsp90 has been shown to associate with a specific subset of cellular proteins involved in signal transduction, including steroid hormone receptors and protein kinases (Nair et al. 1996; Schulte et al. 1996). Hsp90 is known to form characteristic cytosolic complexes with inactive cytosolic steroid hormone receptors which are required for the acquisition of functional competence by the receptors (Catelli et al. 1985; Picard et al. 1990). Evidence suggests that Hsp27 may also function in cell-growth signal transduction. Hsp27 becomes phosphorylated at specific serine residues after treatment of mammalian cells with growth factors or cytokines (Saklatvala et al. 1991; Landry et al. 1992).

The abundance of Hsp90 in the reserve zone (rz) is unique among Hsps and chaperones. The resting cells of the rz are highly responsive to hormones – mainly growth hormone. In addition, it is well established that at puberty high levels of estrogen or testosterone cause the remaining cells of the growth plate cartilage to become hypertrophic, and to undergo the ossification process leading to the growth arrest. Moreover, dexamethasone plays a major role in promoting proliferation in cultured mammalian cell lines of skeletal-cell precursors (Grigoriadis et al. 1988; Poliard et al. 1995).

Among the osteoinductive factors which play a major role in the control of chondrogenesis and osteogenesis and in bone-modeling, TGFβ, BMP-2, BMP-4 and FGF are involved in limb pattern formation and osteogenesis (Carrington et al. 1988; Lyons et al. 1989; Rosen and Thies 1992). More specifically, BMP-2 is strongly expressed in hypertrophic chondrocytes. TGFβ and BMP receptors consist of heterodimers of serine/threonine kinases (Kawakami et al. 1996). FGF interacts with tyrosine kinase FGF receptors, whose mutations dramatically alter endochondral bone osteogenesis (reviewed in Erlebacher et al. 1995; Francomano et al. 1996). Bone-modeling depends on the coordinated activities of chondrocytes and osteoblasts at the chondro-osseous junction. It has been postulated that this process is triggered through paracrine activities and through interactions with extracellular matrix (Cancedda et al. 1995). Results from Shrivastava et al. (1997) and Vogel et al. (1997) (see comment by Schlessinger 1997) demonstrate that different types of collagen of the extracellular matrix bind to and directly activate the 'orphan' tyrosine kinase receptors DDR1 and DDR2 in a kinetic different from that of FGF tyrosine kinase receptors.

Our hypothesis is that Hsps (i.e. Hsp90 and small Hsps) are involved in the proper functioning and/or control of these regulating pathways.

The characterization of the different Hsps and chaperones required during in vitro differentiation of C1 mesodermal cells committed towards chondrogenic or osteogenic fate (Poliard et al. 1995) may open the way to the identification of the role of these proteins in the complex events of endochondral bone formation. The study of chondrogenesis and osteogenesis in animals with one or the other Hsps knocked out would also help us to attribute a precise function to Hsps in the developmental process.

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