Biochemical and proteomic characterization of alkaptonuric chondrocytes

Supplementary Material

Results of the comparative proteomic analysis on "white" and "black" AKU chondrocytes

The most relevant proteins that were found to be differentially expressed in 'white' and 'black' AKU chondrocytes with respect to non-AKU control chondrocytes are described here according to their functional classification.

Metabolism

Within this functional class, the under-expression of dihydropyrimidinase-related protein 2 (DPYL2) in 'white' chondrocytes was noteworthy. DPYL2 is one of the major proteins involved in remodeling of cell cytoskeleton and signal pathway (Inagaki et al., 2001); it is already known to be significantly under-expressed in OA chondrocyte mitochondria (Ruiz-Romero et al., 2009).

In both 'white' and 'black' chondrocytes, we also found an increased abundance of nucleoside diphosphate kinase A (NDKA); similarly, an increase in the isoform NDKB was observed in osteoarthritis (OA) chondrocyte mitochondria (Ruiz-Romero et al., 2009).

Energy

Chondrocytes in articular cartilage have a very limited blood supply (creating nutrient and oxygen gradients) and are likely to face low oxygen tensions. Whereas chondrocytes closest to the synovium are probably exposed to a normoxic tension, cells in the deepest regions of the cartilage may have to face lower oxygen tensions, and some authors suggested that the low rate of aerobic respiration in cartilage cells may be an adaptive response to such an environment (Marcus, 1973). Though cells in the more superficial zones of articular cartilage can rely on mitochondrial oxidative phosphorylation, cells facing anoxia use anaerobic glycolysis as a fundamental way to generate ATP in order to survive and produce ECM (Blanco et al., 2004; Johnson et al., 2004).

A proteomic study on how hypoxia differently modulates normal and OA chondrocytes revealed that OA cells are less reactive to an hypoxic *milieu*, showing important alterations in the abundance of proteins committed to energy production (Ruiz-Romero et al., 2010). Similarly, when we analyzed the expression of proteins involved in energy metabolism, we found an under-expression of glyceraldehyde-3-phosphate dehydrogenase (G3P). The under-expression of this protein was proposed by others as a possible biomarker of OA (Ruiz-Romero et al., 2008). Concomitantly, in 'black' chondrocytes we detected an increased expression of transketolase (TKT). TKT, belonging to the pentose-phosphate pathway, plays a key role in counteracting oxidative stress since it can provide the cells with reducing power. This finding might indicate that 'black' AKU cells experience oxidative stress.

Transcription, synthesis and turnover of proteins

In this functional class we found a general under-expression of identified proteins, including elongation factor 1-alpha 1 (EF1A1), elongation factor Tu (EFTU), and cathepsin D (CATD). CATD is the most abundant cellular endopeptidase in chondrocytes (Ruiz-Romero et al., 2005) and its role in bone metabolism and cartilage turnover was suggested (Handley et al., 2001). Notably, CATD contributes to the proteolytic processing of the core protein of aggrecan in the cartilage, though the potential importance of this enzyme in joint diseases, such as OA, appears relegated to intracellular catabolism (Nakase et al., 2000). In OA, chondrocytes have increased expression and activity of catabolic enzymes such as cathepsin B and D, and both enzymes together with cathepsin K and MMPs help the degradation of ECM. Nonetheless, a significant decrease in chondrocytes positive for CATD was shown in an in vivo model of OA, suggesting that loading forces may modulate such a phenomenon (Bowe et al., 2007) and that CATD may have distinct functions within articular cartilages. In our investigations, we found that levels of CATD were 4.7 and 3.8 times lower in 'white' and 'black' AKU chondrocytes, respectively.

Protein fate

Both 'white' and 'black' AKU chondrocytes showed a significant under expression of identified proteins involved in folding, maturation and transport of proteins. Particularly, lower levels of calreticulin (CALR), 75 kDa glucose-regulated protein (GRP75), protein DJ-1 (PARK7), proteasome activator complex subunit 1 (PSME1) were common to both 'white' and 'black' AKU chondrocytes. Conversely, prolyl 4-hydroxylase subunit alpha-1 (P4HA1) was specifically under-expressed in 'white' AKU chondrocytes whereas protein disulfide-isomerase (PDIA1), transitional endoplasmic reticulum ATPase (TERA) and ubiquitin-conjugating enzyme E2 K (UBE2K) were specifically under-expressed in 'black' AKU chondrocytes with respect to the control. Notably, CALR is required for assembly and quality control of proteins, while PDIA1 has a fundamental role in triple helix collagen formation. GRP75 is a mitochondrial protein with anti-apoptotic functions induced by several stresses, when it is expected to help protein folding (Wadhwa et al., 2002).

Cellular organization

Like in other cells types, in chondrocytes the cytoskeleton consists of microfilaments made of actin, microtubules made of tubulin and intermediate filaments made of a variety of subunits. Cytoskeleton, establishing an intimate connection with ECM, plays an important role in maintaining cell shape and functions (Kim et al., 2003). In our proteomic analysis, we observed that tubulin (TBB5), gelsolin (GELS), tropomyosin alpha-3 chain (TPM3) and WD repeat and FYVE domain-containing protein 1 (WDF1) were under-expressed only in 'black' chondrocytes in comparison to control chondrocytes. Furthermore, we found that annexin A4 (ANXA4) and A5 (ANXA5), vimentin (VIME), and galectin-3 (LEG3) were under-expressed in both 'white' and 'black' AKU chondrocytes.

Among these proteins, it is noteworthy to mention that VIME helps cells tolerate different types of stress allowing a quick cytoskeleton adaptation through sequential degradation and rearrangement processes, being the most sensitive sensor for mechanically-induced stress (Li et al., 2010). The mechanical role of VIME is also postulated basing on its higher expression in chondrocytes from load-bearing cartilages (Langelier et al., 2000) or regions of it undergoing more important deformation

stresses (Eggli et al., 1988). VIME is thus necessary to maintain the chondrocyte stiffness and allow a proper mechanotransduction; changes in VIME organization are considered to play a role in the pathophysiology of OA (in affected cartilages, it is under-expressed (Li et al., 2010; Ruiz-Romero et al., 2009)) and correlate with disease progression (Blain, 2009; Blain et al., 2006; Haudenschild et al., 2010; Lambrecht et al., 2008). In our investigations, VIME levels were 5.2 and 4.1 times lower in 'white' and 'black' AKU chondrocytes, respectively.

Furthermore, GELS regulates chondrocyte architecture and cell-matrix interactions. GELS was found to be lowered in synovial tissues from rheumatoid arthritis (Djouad et al., 2007) or upon IL-1β stress (Wilson et al., 2008) and associated with cytoskeletal remodelling in arthritis synovial cells (Aidinis et al., 2005; Vasilopoulos et al., 2007). We found that GELS levels were 2.0 times lower in 'black' AKU chondrocytes than in the control.

Cell rescue, defence and stress

Like previously indicated by our group in a chondrocytic line used as a model of AKU (Braconi et al., 2010b), levels of proteins involved in cell rescue and defence from stress showed the most interesting alterations. Oxidative stress plays an essential role in the development and progression of OA (Blanco et al., 2004; Gobezie et al., 2007; Johnson et al., 2004) and protection from oxidative stress is fundamental for the avascular tissue of cartilage and highly specialized tissues of joints (Gobezie et al., 2007). Possible relationships among ageing, chronic inflammation and cartilage degradation in OA were reviewed recently (Henrotin et al., 2007); nevertheless, the pathology-related antioxidant status of chondrocytes need to be addressed in greater detail (Henrotin and Kurz, 2007). Importantly, ROS are known to be implicated in the pathogenesis of AKU, like we demonstrated previously in several in vitro models (Braconi et al., 2010a; Braconi et al., 2010b; Tinti et al., 2010).

In this work, we found that AKU chondrocytes showed altered levels of proteins fundamental for the protection from oxidative stress. Lower protein levels were found for the mitochondrial 60 kDa HSP, (CH60), alpha-crystallin B chain (CRYAB), endoplasmin (ENPL), glutathione S-transferase omega-1 (GSTO1), glutathione S-transferase P (GSTP1), heat shock 70 kDa protein 4 (HSP74) and mitochondrial superoxide dismutase (SODM). On the contrary, alpha-crystallin A chain (CRYAA), peroxiredoxin-1

(PRDX1), peroxiredoxin-6 (PRDX6) and serpin H1 (SERPH) were over-expressed both in 'white' and 'black' AKU chondrocytes compared to the control. Notably, PRDX1 is among the proteins released by chondrocyes upon IL-1 stimulation (Catterall et al., 2006), while SODM is a key factor in controlling chondrocyte metabolism and redox-status (De Ceuninck, 2008; Ruiz-Romero et al., 2009). SODM, dismutating peroxide anions, can protect the cells from oxidative damages but lower levels of SODM characterize OA cartilages (Matsumoto et al., 1991; Tiku et al., 2000). In the present work, we showed an important deficiency of SODM in 'white' (-5.7 fold-change) and 'black (-4.6 fold-change) AKU chondrocytes. A similar trend was shown by glutathione S-transferases (GSTs), which have protective roles during oxidative stress (Higuchi, 2004; Vaillancourt et al., 2008) but are lowered in OA (Carlo and Loeser, 2003). GSTO1 has been already addressed as a mediator of inflammation in OA, and several studies associated GSTO1 to stress-induced apoptosis or human pathologies such as Alzheimer's and Parkinson's disease (Ruiz-Laguna et al., 2005) besides OA, where it has been indicated as a disease marker (Ruiz-Romero et al., 2008). GSTs are very important detoxifying enzymes: they can react with and neutralize ROS but also quinones (Higuchi, 2004). This, together with the above-mentioned SODM deficiency, reinforce the hypothesis that AKU cells cannot adequately control ROS- and quinone-mediated toxicity.

Levels of heat shock 70 kDa protein 4 (HSP74) correlates with OA clinical severity (Kubo et al., 1985; Takahashi et al., 1997). Additionally, NO-induced activation of HSPs is thought to suppress apoptosis (Mosser et al., 2000; Terauchi et al., 2003). In our proteomic analysis, we showed a significant under-expression of HSP74 in 'white' (-2.8 fold-change) and 'black' (-5.4 fold-change) chondrocytes with respect to control, suggesting that the lack of this protein may promote NO-induced apoptosis induced AKU chondrocytes.

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