Evidence for lysosomal exocytosis and release of aggrecan-degrading hydrolases from hypertrophic chondrocytes, *in vitro* and *in vivo*

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Summary

The abundant proteoglycan, aggrecan, is resorbed from growth plate cartilage during endochondral bone ossification, yet mice with genetically-ablated aggrecan-degrading activity have no defects in bone formation. To account for this apparent anomaly, we propose that lysosomal hydrolases degrade extracellular, hyaluronan-bound aggrecan aggregates in growth plate cartilage, and that lysosomal hydrolases are released from hypertrophic chondrocytes into growth plate cartilage via Ca^{2+}-dependent lysosomal exocytosis. In this study we confirm that hypertrophic chondrocytes release hydrolases via lysosomal exocytosis *in vitro* and we show *in vivo* evidence for lysosomal exocytosis in hypertrophic chondrocytes during skeletal development. We show that lysosome-associated membrane protein 1 (LAMP1) is detected at the cell surface following *in vitro* treatment of epiphyseal chondrocytes with the calcium ionophore, ionomycin. Furthermore, we show that in addition to the lysosomal exocytosis markers, cathepsin D and β-hexosaminidase, ionomycin induces release of aggrecan- and hyaluronan-degrading activity from cultured epiphyseal chondrocytes. We identify VAMP-8 and VAMP7 as v-SNARE proteins with potential roles in lysosomal exocytosis in hypertrophic chondrocytes, based on their colocalisation with LAMP1 at the cell surface in secondary ossification centers in mouse tibiae. We propose that resorbing growth plate cartilage involves release of destructive hydrolases from hypertrophic chondrocytes, via lysosomal exocytosis.

Key words: Aggrecan, Endochondral bone formation, Growth plate, Hyaluronidase, Hypertrophic chondrocyte, Lysosomal exocytosis

Introduction

The appendicular skeleton is formed upon a cartilage template during endochondral bone formation, within a zone of remodelling cartilage called the growth plate. Endochondral bone formation involves resorption of aggrecan-rich avascular cartilage, followed by deposition of mineral-rich vascularised bone. The complex cellular and signalling processes that regulate endochondral bone formation have been extensively studied and are reviewed elsewhere (Kronenberg, 2003; Wuelling and Vortkamp, 2010). However, the molecular mechanism of how growth plate cartilage is resorbed during bone growth has received less attention.

The most active region of aggrecan resorption in the growth plate is in the hypertrophic zone. As the hypertrophic cells expand to occupy larger volumes (Buckwalter, 1983; Hunziker et al., 1987), the volume of extracellular matrix diminishes and the concentration of aggrecan increases (Matsui et al., 1991; Plaas and Sandy, 1993). However, overall there is a net loss of total aggrecan prior to calcification (Matsui et al., 1991).

Numerous *in vitro* and *in vivo* studies have shown that aggrecan degradation in adult articular cartilage is mediated by members of the A Disintegrin And Metalloproteinase with ThromboSpondin motifs (ADAMTS) family of metalloenzymes, with minor contributions from matrix metalloproteinases (MMPs). Our recent studies investigating aggrecan loss in mice with mutations targeting aggrecanolysis suggest that in contrast to adult articular cartilage, resorption of aggrecan in growth plate cartilage does not rely on ADAMTS enzymes. This conclusion is based on the results of studies with knockin (Little et al., 2007; Little et al., 2005) and knockout (Rogerson et al., 2008; Stanton et al., 2005) mutations in mouse genes designed to disrupt aggrecan catabolism. These mice developed normally with no defects in growth plate morphology and no abnormalities in endochondral bone formation. The lack of a skeletal phenotype in the aggrecan knockin mouse, which is resistant to ADAMTS cleavage (in the interglobular domain) (Little et al., 2007), is particularly informative because there is no mechanism for compensatory cleavage by other ADAMTS enzymes at the primary cleavage site in this mouse. Accordingly, these results suggest that aggrecan loss from growth plate cartilage is not driven by the same proteolytic mechanisms that drive aggrecan loss from mature articular cartilage in joint disease.

In considering alternative mechanisms by which extracellular aggrecanolysis might be achieved in the growth plate, lysosomal enzymes, for example the hyaluronidases, emerged as possible candidates, since aggrecan monomers are immobilised in the

matrix by binding to polymeric hyaluronan. We therefore concluded that lysosomal exocytosis was a novel, potential mechanism for degrading aggrecan in growth plate cartilage.

In addition to specialised cells that release their granular contents by fusion of secretory lysosomes at the plasma membrane, conventional lysosomes in cells such as fibroblasts, epithelial cells and transformed cells can also fuse with the plasma membrane following physiological cell wounding, in a Ca^{2+}-dependent process known as lysosomal exocytosis (Cocucci et al., 2006; Jaiswal et al., 2002; McNeil, 2002; Meldolesi, 2003; Reddy et al., 2001; Wang et al., 2005). Lysosomal exocytosis is a repair mechanism for patching membranes that rupture, for example in response to treatment with pore-forming agents or under conditions of increased biomechanical load. Following membrane disruption, a rapid equilibration of intracellular Ca^{2+} depolymerises the F-actin network to trigger lysosome accumulation near the plasma membrane and lysosomal fusion with the cell membrane to reseal the perforation (Andrews, 2002; Jaiswal et al., 2002; Meldolesi, 2003; McNeil, 2002; Reddy et al., 2001). Thus, resealing of perforated membranes is essential for cells to survive in mechanically active environments.

Regulated lysosomal exocytosis is mediated by integral membrane proteins called soluble NSF [*N*-ethylmaleimide-sensitive fusion protein] attachment protein receptors (SNAREs) present on vesicle (v-SNAREs) or target (t-SNARE) membranes. The v-SNARES and t-SNARES form a highly stable complex that spans the membrane bilayers to mediate membrane fusion and release of vesicle contents. The SNARE protein VAMP8 was originally shown to mediate fusion of late endosomes and lysosomes (Antonin et al., 2000), however it is now clear that VAMP8 also has a role in exocytosis of granules from mast cells (Lippert et al., 2007), platelets (Ren et al., 2007), pancreatic acinar cells (Wang et al., 2004) and macrophages (Pushparaj et al., 2009). Similarly VAMP7, expressed in neuronal (Coco et al., 1999; Oishi et al., 2006) and epithelial (Galli et al., 1998; Sato et al., 2011) cells, is involved in lysosomal exocytosis (Rao et al., 2004).

We postulate that lysosomal exocytosis is an alternative mechanism for releasing aggrecan-degrading hydrolases into growth plate cartilage. Because hypertrophic chondrocytes expand their volume 5–10 fold (Buckwalter, 1983; Hunziker et al., 1987), experiencing stretch-induced load over a period of ~48 hours (Farnum and Wilsman, 1993), we hypothesise that this mechanical challenge causes membrane rupture and lysosomal exocytosis in hypertrophic chondrocytes. In contrast, mature articular chondrocytes are quiescent cells that do not expand or divide; this comparison provides a working model for why lysosomal exocytosis might occur in rapidly expanding hypertrophic chondrocytes in growth cartilage, but not in resting chondrocytes of articular cartilage.

The aim of this study was to test our hypothesis that hypertrophic chondrocytes release lysosomal hydrolases via lysosomal exocytosis. We show that the lysosome-associated membrane protein 1 (LAMP1), normally resident on the internal leaflet of the lysosomal membrane, is detected at the cell surface following *in vitro* treatment of mouse epiphyseal chondrocytes with ionomycin, and that ionomycin treatment induces release of aggrecan-degrading hydrolases in culture. We identify VAMP7 and VAMP8 as the SNARE proteins that could potentially mediate lysosomal exocytosis in hypertrophic chondrocytes. Finally we provide, evidence that lysosomal exocytosis occurs *in vivo* during normal skeletal development indicating that lysosomal hydrolases released from hypertrophic chondrocytes could participate in aggrecanolysis *in vivo*.

Results

LAMP1 is localised at the cell surface of hypertrophic chondrocytes in the tibial growth plate and developing secondary centre of ossification

When lysosomes fuse with the plasma membrane in response to elevated intracellular Ca^{2+}, LAMP1 is exposed on the cell surface (Reddy et al., 2001; Rodriguez et al., 1997). We used anti-LAMP1 antibodies to identify sites of lysosomal exocytosis in chondrocytes present in the developing secondary centre of ossification (Fig. 1A,B,F) and in the hypertrophic zone of the growth plate (Fig. 1C,D), in sections of 6-day old mouse knees (Fig. 1E). LAMP1 was clearly localised at the cell periphery in hypertrophic chondrocytes of the developing secondary centre of ossification (Fig. 1A,B arrows). Similarly, LAMP1 staining in hypertrophic chondrocytes of the growth plate, close to the bony interface, was detected in a punctate pattern at the cell surface (Fig. 1C arrows).

In contrast, LAMP1 staining in chondrocytes of the proliferative zone was restricted to a perinuclear location (Fig. 1C arrowheads). A typical column of chondrocytes (Fig. 1D,E) with perinuclear LAMP1 staining on a proliferative chondrocyte at the bottom of a column (Fig. 1C arrowhead), is shown. The chondrocyte column in the growth plate represents a continuum of cellular differentiation from proliferative cells proximal to the hypertrophic cells distally (Fig. 1D,E) (Farnum et al., 1990). To confirm the cell surface localisation of LAMP1 on hypertrophic chondrocytes, cryosections from 6 day old mouse epiphyses were stained for LAMP1 together with the membrane marker, cell mask orange. Imaging by confocal microscopy of a single cell within the secondary centre of ossification, shows that the plasma membrane marker overlaps spatially with LAMP1 (Fig. 1F). Graphs showing fluorescence intensity for LAMP1 and plasma membrane marker vs distance through the cell (microns) generated by the Leica graphic analyses software, further confirms the cell surface localisation of LAMP1 in mouse hypertrophic chondrocytes (Fig. 1G).

Cell surface LAMP1 labelling in damaged epiphyseal chondrocytes

Treatment with calcium ionophores in the presence of high extracellular Ca^{2+} triggers lysosomal exocytosis in a number of non-specialist secretory cell types including fibroblasts, epipthelial cells, CHO, HeLa and normal rat kidney cells (Jaiswal et al., 2002; Reddy et al., 2001; Rodriguez et al., 1997). When mouse epiphyseal chondrocytes were treated with 10 μM ionomycin, there was an overt increase in LAMP1 labelling at the cell surface compared with vehicle-treated chondrocytes (Fig. 2E,F). FACS analysis (Fig. 2A,B) revealed cell surface LAMP1 on 20% of epiphyseal chondrocytes treated with ionomycin, whereas only 6% of vehicle-treated cells were LAMP1-positive. The ionomycin-induced increase in cell surface LAMP1 was blocked in the presence of 10 mM EGTA (Fig. 2D,H). Electroporation has also been used to induce lysosomal exocytosis in fibroblasts (Huynh et al., 2004). In electroporated epiphyseal chondrocytes we observed a marked increase in cell surface LAMP1 compared with untreated cells (73% compared with 7%) by FACS analysis; the level of cell

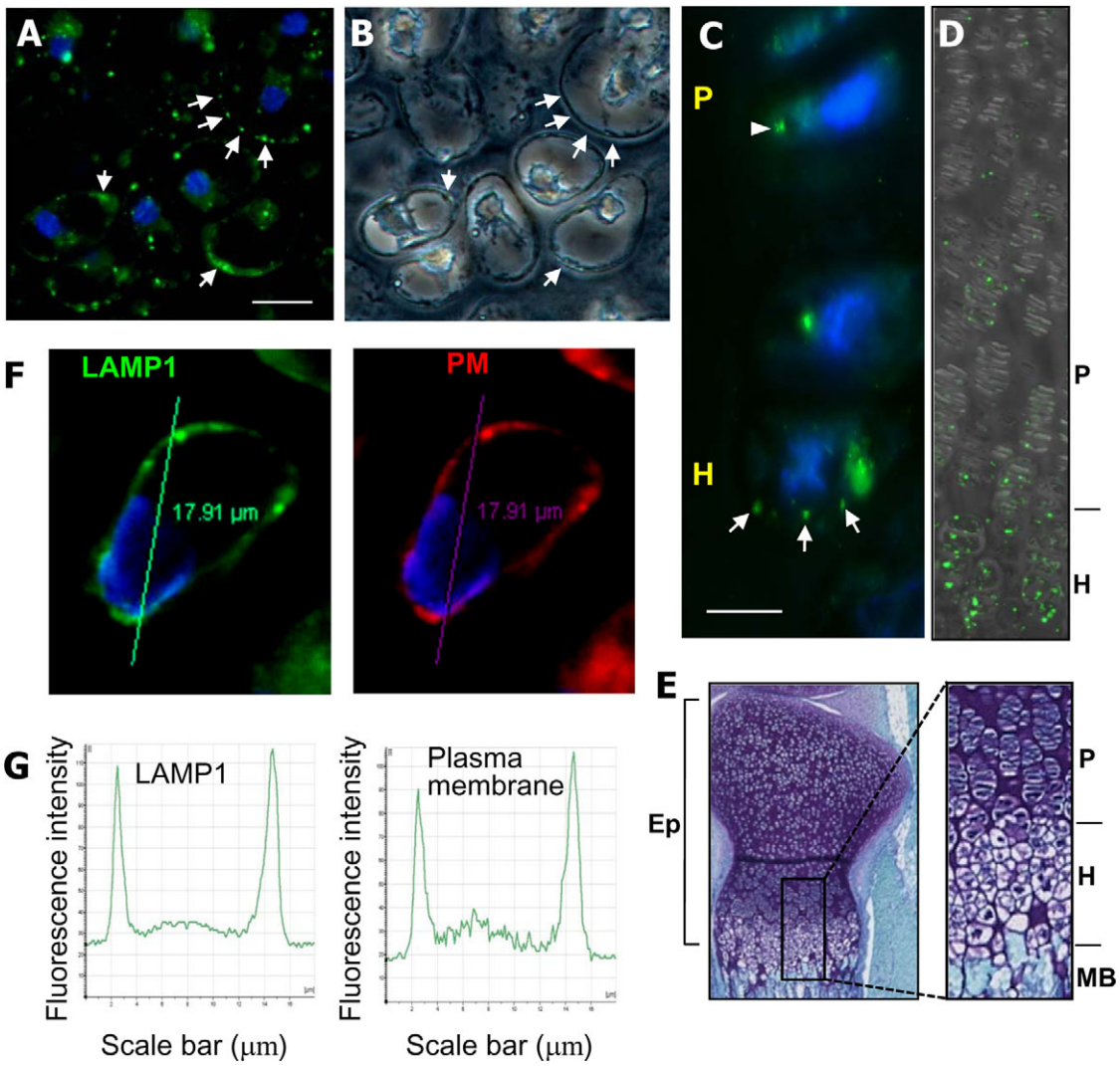

Fig. 1. LAMP1 epitope is present at the cell surface of hypertrophic chondrocytes. Paraffin sections of 6-day old wildtype mouse knees labelled with anti-LAMP1 antibody were imaged by immunofluorescence **(A,C,D)** or phase contrast **(B)** microscopy. LAMP1 is perinuclear in chondrocytes of the proliferative zone (C arrow-heads), but can be seen at the cell surface in hypertrophic chondrocytes of the developing secondary centre of ossification (A arrows), and in the hypertrophic zone of the growth plate (C arrows). Low power images of the epiphysis (Ep) of the distal tibia (E) and expanded sections of the growth plate showing proliferative (P) and hypertrophic zone (H) cells and metaphyseal bone are shown for orientation **(D,E)**. Confocal microscopy of cryosections **(F)** and detection with LAMP1 or cell mask orange confirmed the cell surface localisation of LAMP1 in hypertrophic chondrocytes of the developing secondary centre of ossification. **(G)** Cross section of fluorescence intensity through the cell shown in D. DAPI labels the cell nucleus. Bar=10 µm.

surface LAMP1 was reduced to 39% in the presence of EGTA (supplementary material Fig. S1). Because the cells are not permeabilised before imaging or analysis, LAMP1 can only be detected at the cell surface following lysosomal fusion. Thus, these results suggest that membrane damage to mouse epiphyseal chondrocytes leads to a Ca^{2+}-dependent translocation of LAMP1 to the cell membrane.

Ionomycin induces release of lysosomal cathepsin-D and hexosaminidase from epiphyseal chondrocytes

Release of cathepsin D or β-hexosaminidase from membrane-damaged cells is a standard readout for lysosomal exocytosis. We therefore analysed medium and cell extracts from ionomycin-treated cells for cathepsin D and β-hexosaminidase by Western blot (Fig. 3A,B). Cathepsin D was strongly detected in the conditioned medium of ionomycin-treated cells (Fig. 3A). The cathepsin D-positive bands of 34 kDa and 14 kDa are the heavy and light chains of mature cathepsin D present in lysosomes (Zaidi et al., 2008). No mature cathepsin D bands were detected in the medium of untreated cells, or cells treated with EGTA (Fig. 3A). The cell lysates contained predominantly pro-cathepsin D of Mr 43–48 kDa that is associated with pre-lysosomal compartments within the cell.

We next analysed medium and cell lysates for the presence of hexosaminidase. A 27 kDa hexosaminidase band was strongly detected in the conditioned medium of ionomycin-treated cells, but was undetectable in the conditioned medium of untreated cells, or cells treated with EGTA and ionomycin together (Fig. 3B). This 27 kDa band represents the beta chain of hexosaminidase; some alpha chain (54 kD) was also detected. In untreated cells, or cells treated with ionomycin and EGTA together, β-hexosaminidase signal was detected only in the cell lysates (Fig. 3B). These results confirm that *in vitro* treatment of epiphyseal chondrocytes with ionomycin induces release of

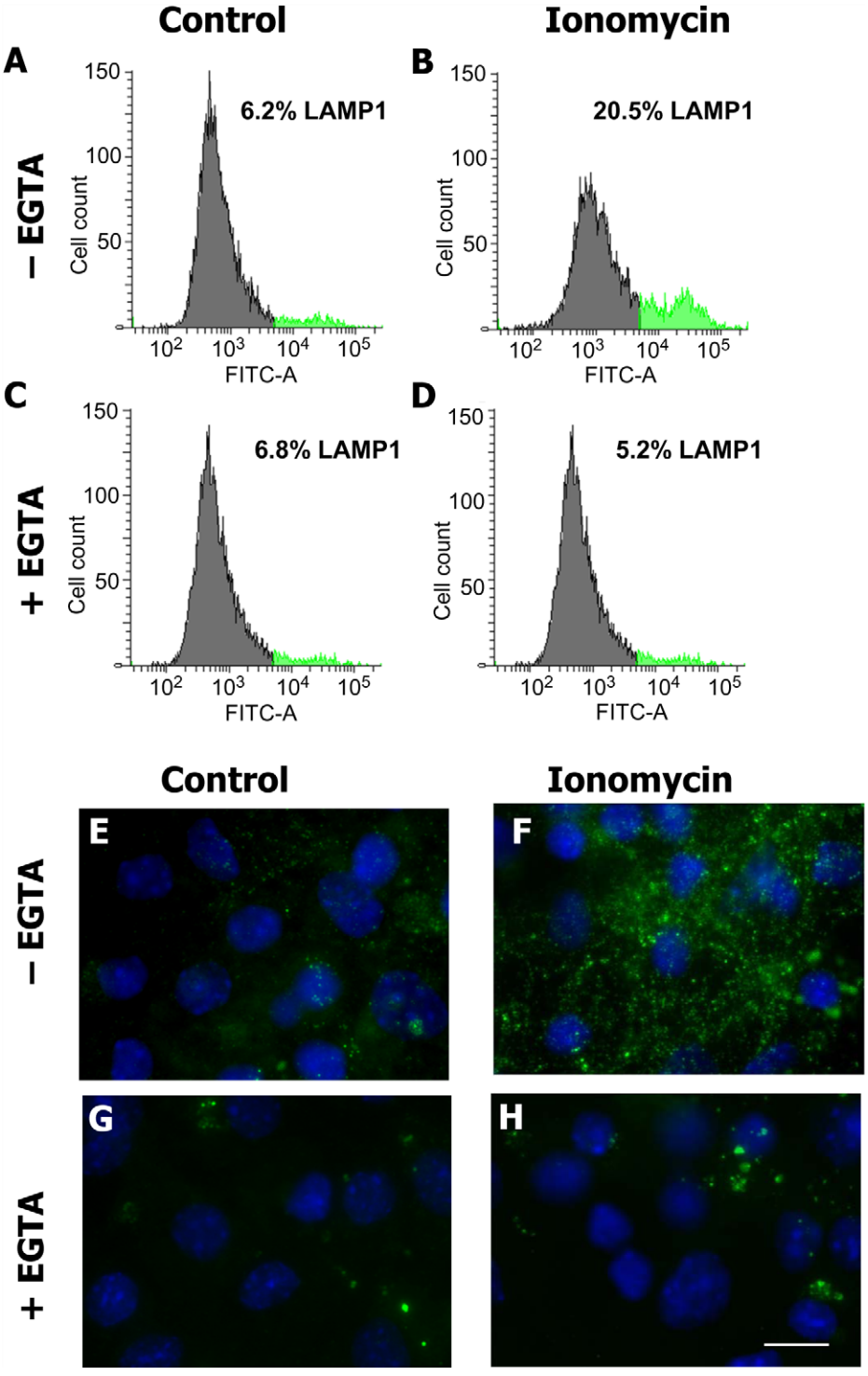

Fig. 2. Ionomycin treatment increases cell surface LAMP1 in epiphyseal chondrocytes. Non-permeabilised mouse epiphyseal chondrocytes treated with 10 µM ionomycin **(B,D,F,H)** and 10 mM EGTA **(C,D,G,H)**, or untreated **(A,E)** were immunolabelled with anti-LAMP1 antibody (E–H) and analysed by FACS (A–D). Under these conditions cell surface but not intracellular LAMP1 is detected. Three independent experiments were done with cells from 3 litters of mice. Bar=10 µm.

lysosomal hydrolases via exocytosis. The concomitant reduction in cathepsin D and hexosaminidase in cell lysates of ionomycin-treated cells correlates well with the increased amounts of these hydrolases in the corresponding conditioned media.

Ionomycin induces release of aggrecan-degrading activity

Since our primary interest in lysosomal exocytosis is its potential to cause degradation of aggrecan in the extracellular matrix, we next examined whether conditioned medium harvested from ionomycin-treated epiphyseal chondrocytes contained enzymes that could degrade exogenous aggrecan substrate. Conditioned medium harvested from cells treated with and without ionomycin and EGTA, was concentrated 20-fold and used as a source of proteinases to degrade exogenous purified aggrecan. The samples were analysed by Western blot using antibodies that recognise carbohydrate epitopes on chondroitinase-digested stubs of chondroitin-4-sulphate (2B6) and chondroitin-6-sulphate (3B3) (Caterson et al., 1985; Christner et al., 1980) on aggrecan. Under

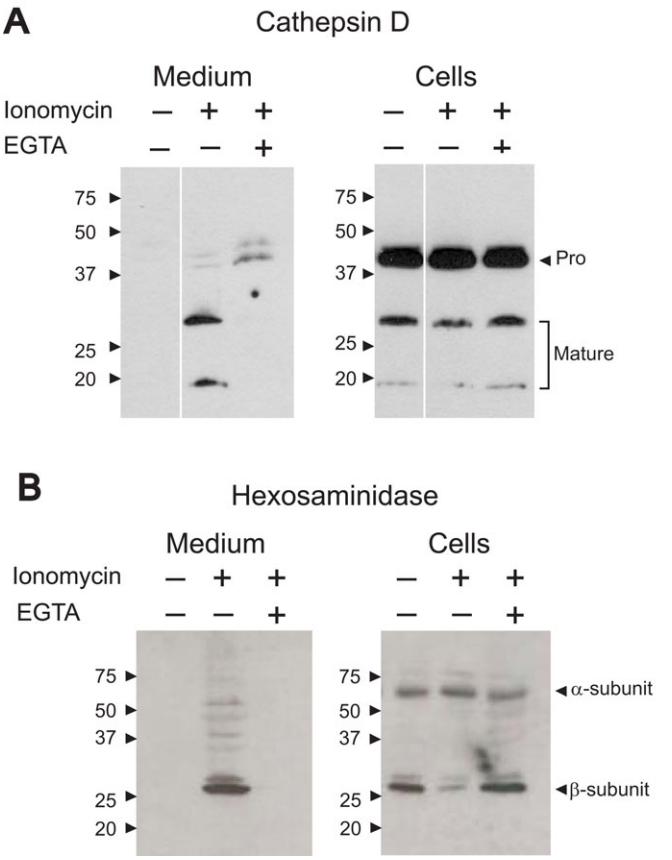

Fig. 3. Ionomycin treatment increases in cathepsin-D and β-hexosaminidase exocytosis in epiphyseal chondrocytes. Conditioned medium and cell lysates from primary mouse epiphyseal chondrocytes treated with or without 10 μM ionomycin and 10 mM EGTA were analysed by Western blotting for cathepsin D **(A)** or β-hexosaminidase **(B)** Three independent experiments were done with cells from 3 litters of mice.

these conditions, only conditioned medium from ionomycin-treated cells contained aggrecan-degrading activity, producing a discrete degradation product at Mr ~130 kDa (Fig. 4A). There was no aggrecan-degrading activity present in conditioned medium from untreated epiphyseal chondrocytes, and the inclusion of EGTA eliminated aggrecan-degrading activity that was otherwise present in cultures treated with ionomycin alone. These results show that one or more aggrecan-degrading hydrolases are released from epiphyseal chondrocytes in response to a calcium ionophore.

We next examined whether ionomycin-treated chondrocytes could degrade and release aggrecan from an endogenous extracellular matrix, assembled during monolayer culture (Fig. 4B). We used the traditional dimethylmethylene blue dye binding assay to measure the concentration of sulphated glycosaminoglycans (Farndale et al., 1982), as a surrogate measure of aggrecan concentration (~90% of aggrecan mass is glycosaminoglycan and aggrecan is overwhelmingly the most abundant proteoglycan in cartilage, by weight). Untreated mouse epiphyseal chondrocytes released 12.8±1.4 μg aggrecan per 3×10^6 cells, whereas epiphyseal cells treated with ionomycin released 20.7±2.9 μg aggrecan per 3×10^6 cells, representing approximately 40% more (Fig. 4B). The difference between the samples with and without ionomycin treatment from 4

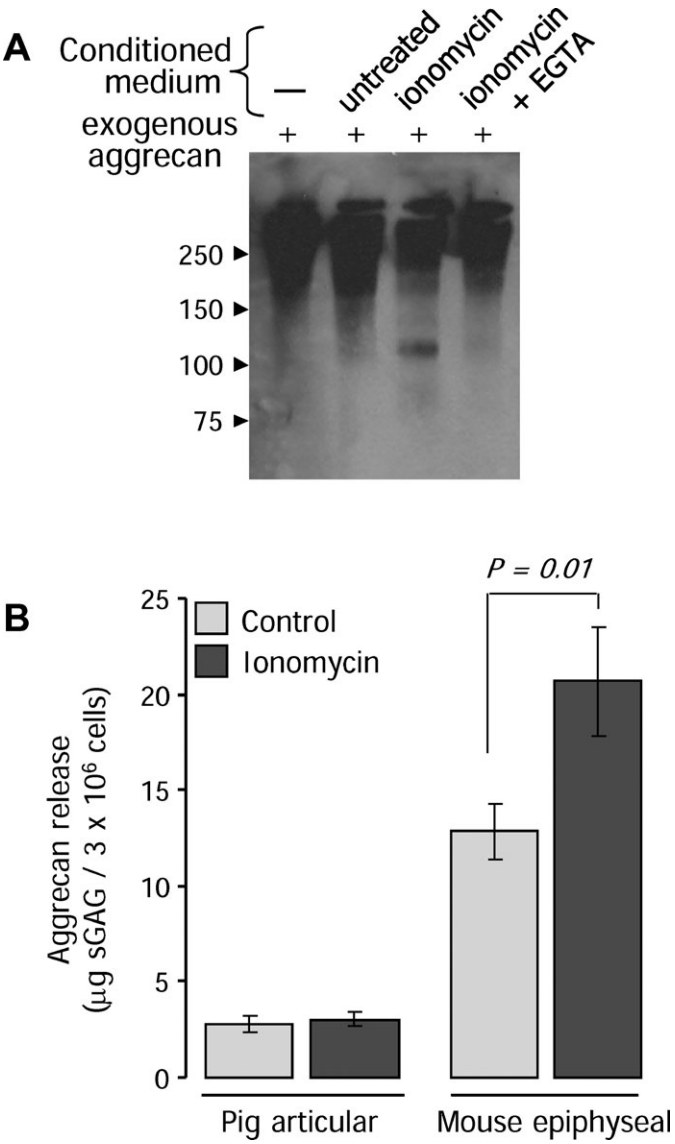

Fig. 4. Conditioned medium from ionomycin-treated chondrocytes promotes endogenous and exogenous aggrecanolysis. (A) Concentrated conditioned medium from epiphyseal chondrocytes treated with ionomycin, ± EGTA, or untreated, was incubated with purified pig aggrecan (A1D1) at pH 4.5 for 16 hours. Aggrecan fragments were detected by Western blot with antibodies 3B3 and 2B6. $n=3$ independent experiments with cells harvested from 3 litters of mice. **(B)** In separate experiments, endogenous aggrecan (glycosaminoglycan) released from monolayer cultures of mouse epiphyseal choncrocytes was detected by the dimethylmethylene blue dye binding assay after treatment ± ionomycin. The results are pooled data from 4 independent experiments from 4 litters of mice, or 3 independent experiments from 3 individual pig joints. Statistical significance was determined with the Students paired t-test ($P=0.01$ for mouse epiphyseal chondrocytes; error bar represents ± sem).

independent experiments was statistically significant with $P=0.01$. We did the same experiment using adult articular chondrocytes isolated from the pig metacarpophalyngeal joint. Ionomycin treatment failed to increase aggrecan release from adult articular chondrocytes (Fig. 4B). Cultures of adult chondrocytes treated with and without ionomycin released approximately 2.9±0.4 μg aggrecan per 3×10^6 cells and were indistinguishable from each other, in 3 independent experiments.

This result could possibly reflect a difference between species, however we expect it is more likely to reflect the difference between hypertrophic chondrocytes in epiphyseal cartilage, and mature chondrocytes in adult articular cartilage. Adult pig cartilage was used for these experiments because mature articular cartilage cannot be harvested in sufficient yield from adult mice without contamination with other joint tissues.

Ionomycin induces release of hyaluronan-degrading activity

In vivo, aggrecan is present as large multimolecular aggregates containing up to 100 aggrecan monomers ($MW_{monomer} \sim 2 \times 10^6$ Da) bound to a single hyaluronan (HA) chain, with each HA-aggrecan interaction stabilised by a small link protein (Neame and Barry, 1993) (Fig. 5A). In addition to proteolysis which degrades the aggrecan core protein adjacent to its linkage with HA, another way to degrade the aggrecan aggregate is to degrade HA (Bastow et al., 2008). There is good evidence that cartilage HA can be degraded *in vitro* by chondrocyte-derived hyaluronidases (Durigova et al., 2011; Sugimoto et al., 2004; Sztrolovics et al., 2002a; Sztrolovics et al., 2002b; Yasumoto et al., 2003), albeit at low levels. To determine whether hyaluronidase activity is present in the 6-day old mouse tibia, we developed a method for in situ hyaluronidase zymography to detect areas of HA loss by overlaying cryosections of mouse joints onto FITC-labelled HA gels. Upon incubation, dark areas of HA lysis were seen in the same regions of the joint that stained positive for LAMP-1 (Fig. 1), namely, the hypertrophic zone adjacent to the metaphyseal bone, and the developing secondary centre of ossification in the middle of the epiphysis (Fig. 5B). No dark areas were detected at the articular surface (Fig. 5B) or in control cryosections incubated with an excess of unlabelled HA.

We next examined the effect of ionomycin treatment on the release of hyaluronidase activity from epiphyseal chondrocytes. Epiphyseal chondrocytes were cultured overnight with exogenous FITC-labelled HA, then treated with or without ionomycin. Untreated cultures showed minimal LAMP1 labelling but abundant FITC-HA that had integrated with the endogenous matrix overnight (Fig. 5C). In contrast, treatment with ionomycin caused a marked increase in cell surface LAMP1 labelling concomitant with a decrease in the intensity of labelling for FITC-HA. These findings suggest a correlation between sites of lysosomal exocytosis and sites of HA resorption from the 6-day mouse tibia.

LAMP1 colocalises with VAMP8 *in vitro* and *in vivo*

To determine which v-SNARE(s) might mediate lysosomal exocytosis in mouse epiphyseal chondrocytes we used double immunofluorescence to detect cell surface LAMP1 with either VAMP7 or VAMP8 in cultured cells. Our results show that the v-SNARE most highly colocalised with LAMP1 in epiphyseal chondrocytes was VAMP8 (Fig. 6). VAMP8 partially colocalised with LAMP1 in perinuclear regions of the cell under basal conditions (Fig. 6A). However, treatment with ionomycin consistently induced a redistribution of VAMP8 and LAMP1 to the plasma membrane where they were colocalised (Fig. 6B). The redistribution of VAMP8 and LAMP1 to the cell periphery was blocked by the addition of EGTA (Fig. 6C). Translocation of VAMP8 and LAMP1 to the cell periphery was also blocked by preincubation with the microtubule depolymerising agent, Nocodazole prior to ionomycin treatment (Fig. 6D). To determine whether VAMP-8 might also have a role in lysosomal exocytosis *in vivo*, we used confocal microscopy to detect LAMP1 and VAMP-8 at the periphery of hypertrophic cells in sections of 6-day old mouse knees (Fig. 7). The results confirm that LAMP-1 and VAMP-8 were colocalised in these sections (Fig. 7), consistent with our hypothesis that lysosomal exocytosis in mouse hypertrophic chondrocytes is potentially mediated, at least in part, by the v-SNARE, VAMP8.

To further investigate the role of VAMP-8 *in vivo*, we compared lysosomal exocytosis in epiphyseal chondrocytes from VAMP-8 null and wildtype mice; in these experiments we were unable to detect differences in the distribution of LAMP-1 immunoreactivity between the two genotypes after treatment with ionomycin (data not shown). In separate experiments we used siRNA to knockdown VAMP8 expression in cultures of wildtype cells, prior to treatment with ionomycin. The results showed that although there was robust knockdown of VAMP8 mRNA to barely detectable levels, there was no corresponding decrease in the release of cathepsin D or hexosaminidase (data not shown), suggesting that VAMP8 might not be the only v-SNARE facilitating lysosomal exocytosis in hypertrophic chondrocytes.

Our finding that ionomycin treatment also induced colocalisation of VAMP7 with LAMP-1 at the surface of hypertrophic chondrocytes (Fig. 6) suggests that VAMP7 might compensate for VAMP8 insufficiency in the VAMP8 null chondrocytes. Although both VAMP8 and VAMP7 were translocated to the cell surface after treatment with ionomycin (Fig. 6B), compared with VAMP8, a significant proportion of VAMP7 immunostaining was also retained intracellularly and in the perinuclear space. The ionomycin-induced redistribution of VAMP7 and LAMP1 to the cell surface was blocked by the addition of EGTA and by pretreatment with Nocodazole (Fig. 6C,D) but again significant VAMP7 was detected in intracellular vesicles. The presence of VAMP7 at the chondrocyte cell surface after ionomycin treatment might explain why ablation of VAMP8 in $VAMP8^{-/-}$ mice, and knockdown of VAMP-8 mRNA by siRNA, failed to block lysosomal exocytosis in mouse chondrocytes, and suggests that VAMP7 might compensate for the loss of VAMP8, *in vitro* and *in vivo*.

Overall, our results suggest that lysosomes in hypertrophic chondrocytes are a potential source of aggrecan-degrading hydrolases. Our results are also consistent with the hypothesis that VAMP7 and VAMP8 have a role in lysosomal exocytosis in hypertrophic chondrocytes and thereby contribute to cartilage resorption in the growth plate.

Discussion

We have used the classical methodologies of ionomycin treatment, cell surface LAMP1 immunostaining and exocytic release of cathepsin D and hexosaminidase, to demonstrate that mouse epiphyseal chondrocytes can undergo Ca^{2+}-dependent lysosomal exocytosis. Since conventional lysosomes in a number of cell types use exocytosis to reseal disrupted membranes *in vitro*, it was not surprising that we were able to replicate these findings in cultured epiphyseal chondrocytes. Our confocal microscopy data now provide new evidence to suggest that lysosomal exocytosis has a physiological role in endochondral ossification *in vivo*. Our findings are consistent with our hypothesis that hypertrophic chondrocytes (in contrast to non-hypertrophic chondrocytes of

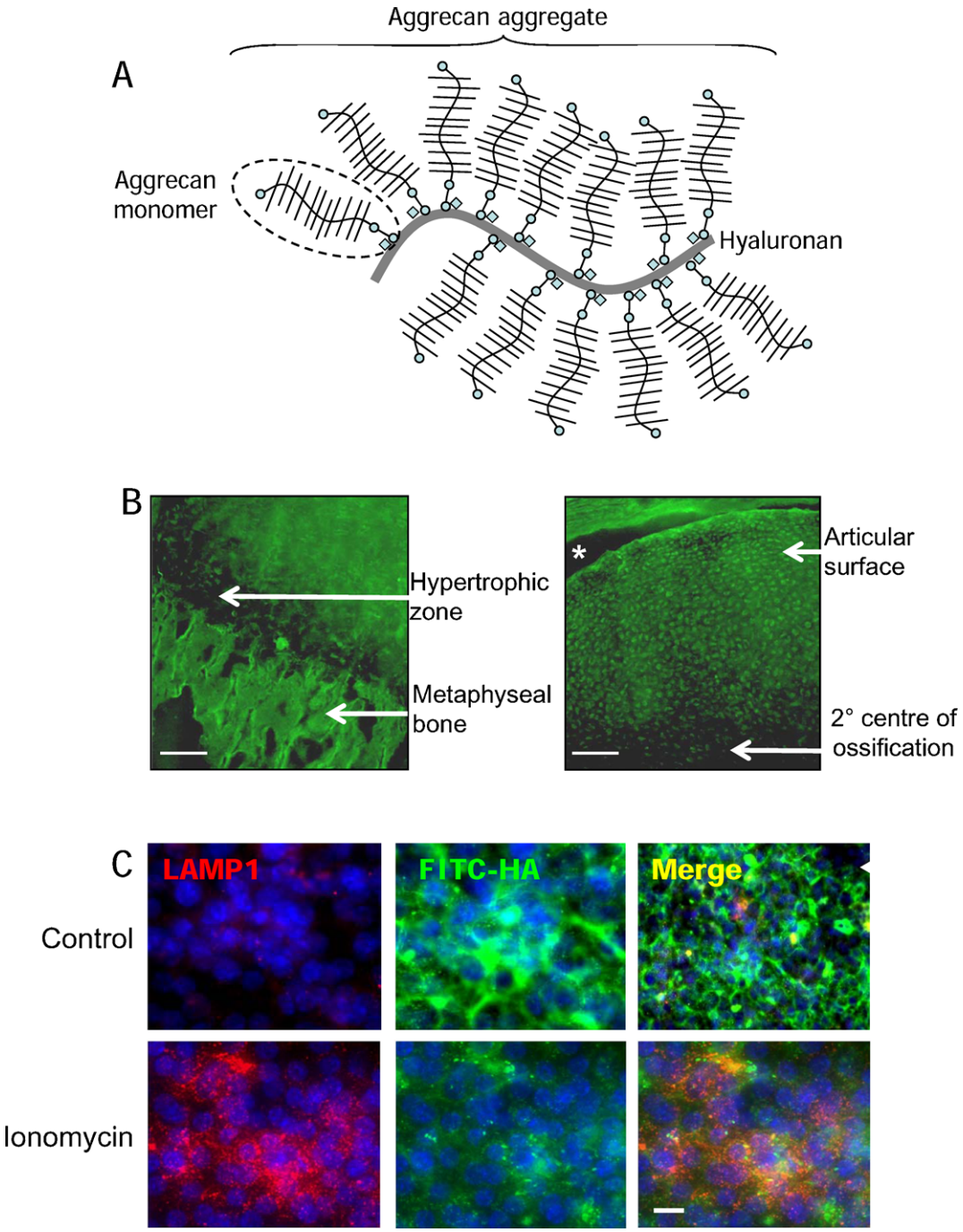

Fig. 5. Ionomycin-induced release of hyaluronan-degrading activity from mouse epiphyseal chondrocytes. (A) Schematic of an aggrecan aggregate comprising 14 aggrecan monomers bound to a single HA polysaccharide, stabilised by link protein (diamonds). Aggrecan monomers comprise a core protein with three globular domains (circles) and numerous chondroitin sulphate chains covalently attached. **(B)** Cryosections of 6-day mouse tibiae were overlaid onto FITC-HA/agarose gels and incubated to develop dark areas of hyaluronan lysis corresponding to regions of hyaluronidase activity in the hypertrophic zone, and secondary centres of ossification in the middle of the epiphyses. No hyaluronidase activity was present in metaphyseal bone or at the articular surface. Asterisk marks the joint space. Bar=50 μm. **(C)** Mouse epiphyseal chondrocytes cultured overnight in the presence of exogenous FITC-HA were treated with or without ionomycin then analysed by fluorescence microscopy for LAMP1 and FITC-HA labelling. Bar=10 μm.

adult and juvenile cartilage) are well adapted for lysosomal exocytosis for the following reasons: hypertrophic chondrocytes expand their volume 5–10 fold over a period of only 2 days and are therefore under stretch-induced load, 2) hypertrophic chondrocytes have a requirement for membrane expansion in order to achieve this increase in cell volume that occurs within a few days, 3) lysosomal hydrolases are an alternative source of aggrecan-degrading activity in growth plates of mice with gene mutations targetting aggrecanolysis (Glasson et al., 2005; Little et al., 2007; Little et al., 2005; Rogerson et al., 2008; Stanton et al., 2005).

The motivation for this study was based on our working hypothesis that, when aggrecanase activity is specifically ablated

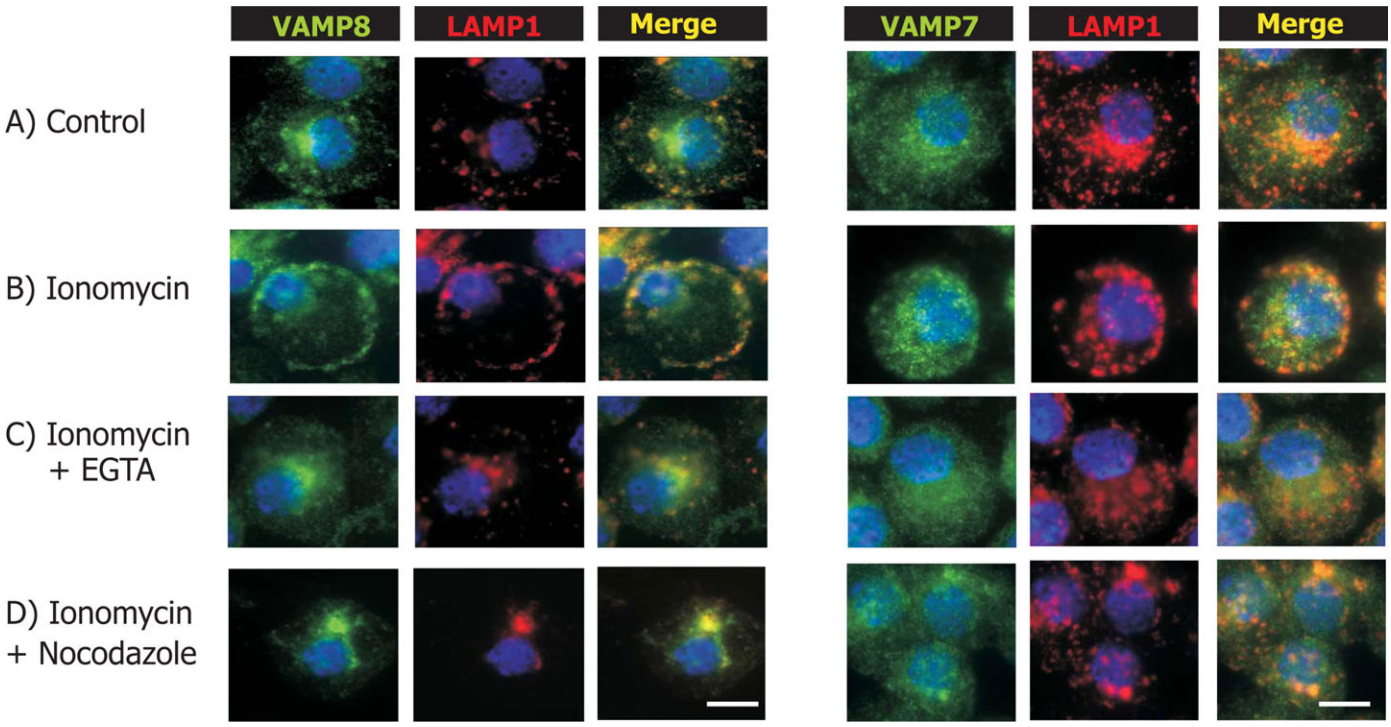

Fig. 6. VAMP8 and VAMP7 colocalise with LAMP1 in epiphyseal chondrocytes under basal conditions and after ionomycin-induced translocation to the cell surface. Epiphyseal chondrocytes were immunolabelled for VAMP7 or VAMP-8 (green) and LAMP1 (red) after treatment with ionomycin **(B)**, ionomycin in the presence of 10 mM EGTA **(C)**, ionomycin after pre-treatment with 3 µM nocodazole **(D)** and untreated cells **(A)**. Three independent experiments were done with cells from 3 litters of mice. Bar=10 µm.

in genetically modified mice, lysosomal hyaluronidases facilitate aggrecan loss from resorbing cartilage by depolymerising the HA backbone that binds aggrecan monomers into multimolecular aggrecan aggregates (Fig. 5A). Clearly, one prerequisite for our hypothesis is confirmation that hypertrophic chondrocytes are equipped to release these enzymes from lysosomes. In this study we have provided evidence that hypertrophic chondrocytes release their lysosomal contents in response to membrane disruption and high extracellular Ca^{2+} *in vitro*, and more importantly, that this process occurs *in vivo* in normal, unstimulated conditions. Our data therefore suggest that under tissue-specific conditions (growth plate hypertrophy), lysosomal exocytosis plays a role in organ-specific (long bone) growth and maturation, using a ubiquitous cellular event that is neither tissue, nor organ specific.

The intrinsic weight-bearing properties of cartilage are conferred by the high, fixed charge density provided by the high concentration of aggrecan aggregates. Binding to HA is essential for immobilising aggrecan in cartilage, and degrading the aggregate structure by either proteinases or hyaluronidases is essential for aggrecan turnover. Because of their vast size, neither aggrecan monomers nor aggregates, can be internalised by chondrocytes unless there is prior cleavage by proteinases (Embry and Knudson, 2003). Aggrecanase activity liberates the bulk of the aggrecan monomer from its HA anchor, causing rapid aggrecan loss into the joint space. Despite the lysosomal location of hyaluronidases, and despite the lack of robust molecular evidence of hyaluronidase activity due to lack of specific assays or inhibitors, there is considerable circumstantial evidence for extracellular HA degradation in cartilage (Buckwalter et al., 1987; Campo and Romano, 1986; Chockalingam et al., 2004; Durigova et al., 2011; Fosang et al., 1991; Plaas and Sandy, 1993; Sztrolovics et al., 2002a; Sztrolovics et al., 2002b; Yoshida et al., 2004). However, the mechanism by which hyaluronidases arrive in the cartilage matrix has not previously been addressed.

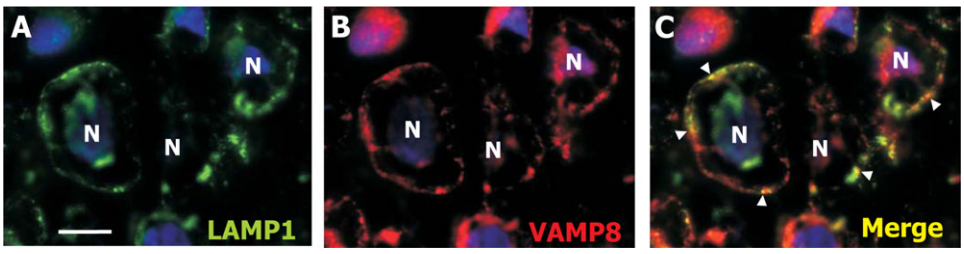

Fig. 7. VAMP8 colocalises with LAMP1 at the plasma membrane on hypertrophic chondrocytes within the developing secondary centre of ossification. Sections of 6-day old mouse knees were immunolabelled for LAMP1 (green) (**A**) and VAMP8 (red) **(B)**, and the staining visualised by confocal microscopy. VAMP8 and LAMP1 colocalised at the cell periphery adjacent to the cartilage matrix (arrow heads, panel **C**). Three independent experiments were done with cells from 3 litters of mice. N=nucleus, Bar=10 µm.

Work with adult articular cartilage explants show that catabolic stimuli induce concomitant release of HA, link protein and aggrecan G1 domains into conditioned media (Chockalingam et al., 2004; Durigova et al., 2011; Fosang et al., 1991; Sugimoto et al., 2004). Other *in vitro* studies in growth cartilages have also found evidence of degraded HA, for example, intact aggrecan monomers are released from fetal cartilage and HA released from IL-1-stimulated fetal cartilage explants is smaller than HA released from unstimulated cultures (Plaas and Sandy, 1993; Sztrolovics et al., 2002a; Sztrolovics et al., 2002b). Furthermore, the size of HA-aggregates in the hypertrophic zone is reduced compared with other zones of the growth plate, due to a decrease in the length of the HA polymer and a consequent decrease in the number of monomers attached (Buckwalter et al., 1987; Campo and Romano, 1986). These studies provide evidence for degradation of HA-aggrecan aggregates, particularly in growth cartilage, but they do not reveal the mechanism. Our present study now provides a viable mechanism whereby hyaluronidase activity could become available in the growth plate matrix to participate in aggrecanolysis. Of course other lysosomal proteinases, including cathepsins, could also be involved in growth plate aggrecanolysis. The exact identity of the hydrolases involved remains to be determined.

Finally, our finding that the v-SNAREs VAMP8 and VAMP7 potentially mediate lysosomal exocytosis in hypertrophic chondrocytes is intriguing in the context of osteoarthritis (OA). Some cartilage changes in OA are thought to recapitulate features of endochondral ossification. In particular OA chondrocytes are thought to overcome maturational arrest and revert to a more foetal phenotype including cellular hypertrophy, expression of well characterised hypertrophy markers, followed by mineralisation and cell death (Drissi et al., 2005). Although OA lacks the rampant synovial inflammation characteristic of rheumatoid arthritis, it is nevertheless accompanied by moderate levels of joint inflammation. OA chondrocytes express inflammatory cytokines including IL-1, TNFα and IL-6. Intriguingly, VAMP8-positive vesicles in macrophages of wildtype mice have recently been shown to colocalise with TNFα, and in the same study, TNFα release from macrophages was inhibited in VAMP8-deficient mice (Pushparaj et al., 2009). If VAMP8 and TNFα are similarly colocalised in hypertrophic chondrocytes, it is tempting to speculate that lysosomal exocytosis in hypertrophied OA chondrocytes might represent a new and unprecedented disease mechanism in osteoarthritic disease.

Materials and Methods

Reagents

Collagenase II was from Worthington Biochemical (Lakewood, NJ, USA). Ionomycin (cat NO.407952) was from Calbiochem, CA, USA. Monoclonal antibodies anti-LAMP1 (1D4B) from the Developmental Studies Hybridoma Bank, Iowa US, anti-chondroitin-4-sulphate (2B6) from Seikagaku Corporation, Tokyo, Japan and chondroitin-6-sulphate (3B3), a gift from Prof. Bruce Caterson, Cardiff Wales, were used in this study in addition to rabbit polyclonal antibodies anti-cathepsin D (EPR3057Y) and anti-VAMP7 (SYBL1) from Abcam Cambridge UK, anti-VAMP8 (Endobrevin) from Synaptic Systems Göttingen Germany, anti-hexosaminidase (alpha and beta chains) from Proteintech IL USA. DAPI, cell mask orange and the fluorescently tagged secondary antibodies anti-rat ALEXA 488, anti-mouse ALEXA 488 and anti-rabbit ALEXA 594 were from Molecular Probes, OR, USA. Secondary anti-mouse and anti-rabbit HRP conjugated antibodies from Dako, Glostrup, Denmark were used for Western blotting. Glass chamber slides and tissue culture plates were from Nunc (Labtech, NY, USA). ECL Western Blotting reagent was from Amersham (Buckinghamshire, UK). Hybond-PVDF membrane and 3 kD cut-off spin columns were from (Millipore, MA, USA). Shark cartilage chondroitin sulphate, umbilical cord-derived hyaluronan, dimethylsulphoxide (DMSO), acetalydehyde, cyclohexylisocyanide, and fluorescein amine were from Sigma, MO, USA. Chondroitinase ABC was from MP Biomedicals, OH, USA. Keratanase I and keratanase II were from Seikagaku Corporation, Tokyo, Japan. Nocodazole was from EMD Chemicals, New Jersey, USA.

Isolation of primary epiphyseal chondrocytes from 6-day old mouse knees

All procedures involving mice were approved by the local Institutional Animal Ethics Committee. Proximal tibial and distal femoral epiphyses of 6–8 day old C57BL/6 mice were harvested under a dissecting microscope taking care to remove as much extraneous fibrous material as possible (Rogerson et al., 2010; Stewart et al., 2006). Pooled epiphyses from a single litter were incubated for 1 hour at 37℃ with 0.25% trypsin/EDTA to further remove loose fibrous tissue, then washed three times in PBS. Epiphyseal chondrocytes were released from the cartilage matrix by overnight digestion at 37℃ with 2 mg/mL Collagenase II in DMEM containing 100 U/mL penicillin and 100 U/mL streptomycin, 2 mM L-glutamine and 20 mM HEPES (referred to as medium) supplemented with 5% fetal bovine serum (FBS). These epiphyseal chondrocytes were a mixed population of juvenile cells including hypertrophic chondrocytes from the presumptive secondary centre of ossification that begins to mineralise at ~10 days of age in the mouse knee. After collagenase digestion the cells were washed extensively with medium containing 10% FCS to inactivate the collagenase. The typical yield was $\sim 1 \times 10^6$ cells per mouse. Pig articular chondrocytes were isolated from the metacarpophalyngeal joints of young adult pigs as described previously (Stanton et al., 2002).

Ionomycin treatment of mouse epiphyseal chondrocytes

Ionomycin treatment was used to induce Ca^{2+}-dependent lysosomal exocytosis. Briefly, epiphyseal chondrocytes were cultured in either 8-well glass chamber slides (3×10^5 cells/300 µL) for immunofluorescence, 6-well plates (1×10^6 cells/2mL) for FACS analysis, or 24-well plates (2×10^6 cells/500 µL) for harvesting of conditioned medium for Western blot analysis. Cells were deprived of serum for 16 hours prior to treatment with 10 µM ionomycin, or vehicle, in serum-free DMEM supplemented with 1 mM $CaCl_2$. To block calcium-dependent exocytosis, 10 mM EGTA was added to the cells 10 minutes prior to ionomycin treatment. In some experiments, cells were treated with 3 µM Nocodazole for 1 hour prior to ionomycin treatment.

For FACS analyses, single cell suspensions recovered by treating with 0.25% trypsin for 5 minutes at 37℃ were incubated at 6℃ with 0.6 µg/mL anti-LAMP1 antibody in PBS for 30 mins followed by anti-rat ALEXA 488 secondary antibody in 1% BSA/PBS for 30 minutes, then 10,000 cells were sorted by flow cytometry on a FACS LSRII, (Becton and Dickinson).

Immunofluorescence of non-permeabilised and permeabilised epiphyseal chondrocytes

LAMP1 epitope at the cell surface of non-permeabilised cells was detected with 0.6 µg/mL anti-LAMP1 antibody as described above. In other experiments, fixed cells permeabilised with 100% methanol at −20℃ for 5 minutes, were blocked with 3% BSA/PBS for 30 minutes, then double labelled with 0.6 µg/mL anti-LAMP-1, and either anti-VAMP7 or anti-VAMP8 (1:500 dilution) overnight at 6℃. The next day cells were incubated with fluorescently labelled secondary antibodies and DAPI stain for 1 hour at room temperature, then viewed under a Zeiss AXIO Imager M1 fluorescence microscope. Images were captured using an AXIO digital camera and associated Zeiss Axiovision Rel. 4.7 software.

Immunohistochemistry of mouse epiphyseal cartilage

Knees from 6-day old mice imbedded in OCT compound (Tissue-Tek) were immediately snap-frozen in liquid nitrogen and cryosectioned to 6 µM thickness. Cryosections were fixed with 4% paraformaldehyde for 10 minutes, blocked with 3% BSA in PBS for 1 hour, then immunostained for LAMP1, VAMP8 and DAPI as above. Sections were mounted with FluorSave reagent under a glass coverslip, (size 1.5). The plasma membrane stain Cell Mask Orange (0.5 µg/mL) was added to sections for 10 minutes.

FITC labelling of hyaluronan

FITC labelling of HA was adapted from a previously described method (de Belder and Wik, 1975). Briefly, 100 mg of umbilical cord hyaluronan dissolved in 80 mL of water was diluted with 40 mL DMSO, then 50 µL acetaldehyde, 50 µL of cyclohexylisocyanide and 50 mg of flouresceinamine was added. The solution was adjusted to pH 2.5 with HCl and agitated gently for 16 hours until a dark brown solution was formed. The FITC-labelled hyaluronan was precipitated from the solution with 3 volumes of ethanol. The precipitate was washed 3 times in fresh ethanol, dissolved in water and further dialysed against water for 48 hours. The FITC-HA solution was freeze dried and stored as a dry powder at −20℃.

In situ hyaluronidase zymography

FITC-labelled HA was prepared at 1 mg/mL in water and mixed with an equal volume of warmed 1% agarose in 50 mM Tris-HCl pH 7.2. The melted agarose/FITC-HA solution was spread evenly onto pre-warmed microscope slides and allowed to dry horizontally at room temperature. Each slide was examined by fluorescence microscopy to ensure an even coating of fluorescent substrate on the slide. Cryosections (5–10 microns thick) were placed onto the agarose/FITC-HA-coated slides, covered with 50 mM sodium acetate buffer, pH 5.5 and incubated at 37℃ for 30 minutes. Areas of hyaluronidase activity were identified as dark areas within the fluorescent coating. Parallel sections were co-incubated with an excess of unlabelled HA (100 μg/mL) as negative controls.

Pericellular matrix labelling with FITC-HA

Mouse epiphyseal chondrocytes were grown in 8-well chamber slides in medium with 10% serum for 5 days, before removing the serum, and adding FITC-HA (100 μg/mL) in serum-free medium overnight. The next day cells were treated with 10 μM ionomycin or vehicle control for 30 minutes in serum free medium, then immunolabelled for cell surface LAMP1 followed by anti-rat ALEXA594 secondary antibodies in 1%BSA-PBS for 1 hr at room temperature. Washed cells were mounted with FluorSave reagent and glass coverslips. The change in pericellular-associated FITC-HA and cell surface LAMP1 was monitored by fluorescence microscopy.

Western blotting

For Western blotting cell extracts were prepared by lysis of $\sim 2 \times 10^6$ cells in 200 μL of RIPA buffer (150 mM NaCl, Tris HCl 50 mM, 1% NP40, 0.1% SDS, 0.5% deoxycholate and 2 mM DTT) and homogenised by 25 aspirations through a 21G needle. Proteins in 500 μL of conditioned medium were precipitated overnight with 9 volumes of 100% ethanol at −20℃. The precipitate was recovered by centrifugation at 13,000 rpm for 10 minutes, washed twice with 75% ethanol then resuspended in 12.5 μL RIPA buffer for electrophoresis on 10% SDS gels and transferred to PVDF membrane. The membranes were blocked in 5% skim milk-TBS-0.1% Tween at 4℃ overnight then probed with anti-cathepsin-D or anti-hexosaminidase antibodies, and anti-rabbit-HRP secondary antibody, followed by detection on film with ECL reagent.

Degradation of exogenous aggrecan by lysosomal hydrolases released from ionomycin-treated epiphyseal chondrocytes

To investigate ionomycin-induced release of aggrecan-degrading activity from epiphyseal cells, we used pig aggrecan purified by associative and dissociative (A1D1) CsCl density gradient centrifugation (Heinegard and Sommarin, 1987) as a substrate, incubated with concentrated conditioned medium from epiphyseal cell cultures as a source of enzyme. Conditioned medium was concentrated 20-fold on a 3 kDa cut-off spin column, then incubated with 20 μg purified aggrecan in buffer containing 1 mM EDTA, 1 mM dithiothreitol, 100 mM Tris-HCl pH 4.5 for 16 hours at 37℃ in a total volume of 30 μL. The samples were adjusted to neutral pH and deglycosylated in 0.1 M Tris/Acetate buffer pH 7.2 with chondroitinase ABC (125 mU/ml), Keratanase I (62.5 mU/ml) and Keratanase II (6.25 mU/ml), then aliquots containing 0.4 μg of aggrecan were electrophoresed on 5% Fairbanks polyacrylamide gels (Fairbanks et al., 1971) for Western blot analysis with antibodies 3B3 and 2B6 that detect residual stubs of chondroitin-4-sulphate and chondroitin-6-sulphate after deglycoslyation.

Degradation of endogenous aggrecan following treatment of chondrocytes with ionomycin

The concentration of sulphated glycosaminoglycan released into conditioned medium of cultured chondrocytes was determined using the 1,9-dimethylmethylene blue dye binding assay (Farndale et al., 1982), adapted for microtitre plates (Ratcliffe et al., 1988). Chondrocytes were cultured in 6-well plates for 7 days in DMEM with 10% FCS in order to accumulate an aggrecan-rich extracellular matrix. The cells were then deprived of serum for 16 hours prior to treatment with 10 μM ionomycin in the presence of 1 mM $CaCl_2$ in 1 mL serum-free medium. Mouse epiphyseal chondrocytes were seeded at 5×10^5 cells per well and the medium from 6 wells was pooled for analysis. Adult pig articular chondrocytes, which are significantly smaller than the mouse epiphyseal cells, were seeded at 3×10^6 cells per well and the medium from 6 wells was pooled for analysis. Pooled medium concentrated 12-fold by dialysis and freeze-drying was assayed for sulphated glycosaminoglycans, using shark cartilage chondroitin sulphate as standards.

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Competing Interests

The authors declare no competing interests.

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