Production of cartilage link protein by human granulosa-lutein cells

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Abstract

Link protein (LP), an extracellular matrix protein in cartilage, stabilizes aggregates of hyaluronic acid (HA) and proteoglycans, including aggrecan and inter-α-trypsin inhibitor (ITI). We have shown previously that cartilage LP is present in the maturing rat and mouse ovary. In the present study, we have employed immunohistochemistry to examine the anatomical distribution of cartilage LP in the human ovary. The expression of cartilage LP was selectively detected in the cells within the granulosa compartment of the preovulatory dominant follicle. The HA-positive granulosa-lutein cells were found to be a cartilage LP-positive subpopulation. We subsequently studied the in vitro expression of cartilage LP in cultured human granulosa-lutein cells obtained at oocyte retrieval for in vitro fertilization. Analysis of cultured cells by enzyme-linked immunoaffinity assay, Western blotting and immunofluorescence microscopy revealed that gonadotropin stimulates cartilage LP production. Time-course studies indicated that the cartilage LP production was induced as early as with gonadotropin stimulation for 2 h, and the effect was sustained up to 8 h. Western blot analysis further revealed the presence of the macroaggregates of HA, ITI and cartilage LP in the gonadotropin-stimulated granulosa-lutein cell extracts. Collectively, the present results raise the possibility that cartilage LP forms extracellular structures that may have a regulatory function in the developing follicle in the human ovary.

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Introduction

Analysis of bioactive substances produced by granulosa cells or oocytes is one approach to understanding the biological features on cumulus cell–oocyte complex (COC) expansion during the preovulatory period. In the last few years, several studies have been made of the cellular system that controls the initiation and progression of COC expansion: several hormones, cytokines and growth factors have been reported to regulate COC expansion in vitro and in vivo (Salustri et al. 1989, Camaioni et al. 1993, Chen et al. 1993, 1994, Hirashima et al. 1997, Tirone et al. 1997, Elvin et al. 1999, Kobayashi et al. 1999a, Otsuka et al. 2000, Carrette et al. 2001). The extracellular matrix of cumulus cells consists of a number of interacting macromolecules that are unique to gonadotropin-stimulated mature ovaries (Chen et al. 1993, Hirashima et al. 1997, Zhou & Kimata 2001). Some of these bioactive substances are reported to be hyaluronic acid (HA), inter-α-trypsin inhibitor (ITI) and oocyte-derived factors. The most studied group of these molecules are those that make up the complexes involving HA and proteoglycans including ITI, since an interacting protein for HA has been recently discovered and identified as a serum-derived 240 kDa ITI (Salustri et al. 1989, Chen et al. 1992, 1994, Camaioni et al. 1993, Huang et al. 1993, Jessen et al. 1994, Zhao et al. 1995). A growing body of evidence has accumulated indicating that HA and ITI accumulate in mature follicles just before ovulation (Salustri et al. 1989, Camaioni et al. 1993, Chen et al. 1993, 1994, Hirashima et al. 1997, Kobayashi et al. 1999a), that HA synthase 2 and its mRNA are present in isolated granulosa cells (Elvin et al. 1999), and that ITI could function as an HA-rich matrix stabilizer on the cumulus cells of maturing ovary, implying involvement of an HA–ITI system in COC expansion (Fulop et al. 1997).

Human cartilage link protein (LP) is a glycoprotein of 40–49 kDa (Poole et al. 1984, Nguyen et al. 1989, Roughley et al. 1993, Neame & Barry 1994); it is the major non-collagenous protein found in cartilage extracellular matrix and stabilizes the binding between HA and proteoglycans, including aggrecan and possibly proteins of the ITI family, and influences the spacing of the monomers along the HA filament. Cartilage LP has been reported to be present not only in cartilaginous tissues but also in aorta (Vijayagopal et al. 1985), eye (Tsonis & Goetinck 1988), skin (Bertheim & Hellstrom 1994) and in synovium (Hoedt-Schmidt et al. 1993), based largely on immunological criteria. However, the structural relationships among these species and their functions have not been fully established.
Although the molecular basis for the formation of the ternary complex involving HA, ITI and cartilage LP in COC expansion is not known, there is evidence that this type of interaction is similar to that observed in the extracellular matrix of cartilage (for example, HA–aggrecan–cartilage LP) (Hoedt-Schmidt et al. 1993). These findings allowed us to speculate on the biochemical evidence suggesting that the ternary complex involving HA, ITI and cartilage LP is an important component of the extracellular matrix of luteinized granulosa cells.

The localization study of cartilage LP by the immuno-histochemical approach using a specific antibody has been reported so far only for rat (Kobayashi et al. 1999b) and mouse (Sun et al. 1999) ovary. There is no information on the expression and localization of cartilage LP molecules in human ovary. For a better understanding of the relationship between the HA–ITI–cartilage LP system and COC expansion, analysis of the expression of these components in individual cells would be required. Our aim in this study was (1) to examine the localization of LP in the human ovary, (2) to determine the expression of cartilage LP in gonadotropin-stimulated and unstimulated human granulosa-lutein cells in culture by a newly developed specific enzyme-linked immunosorbent assay, Western blot and by immunofluorescence microscopy, and (3) to determine the formation of HA–ITI–cartilage LP complexes in the cultured granulosa-lutein cells using immunoblotting.

Materials and Methods

Tissue collection and follicular characterization

Human ovarian tissue (n=17) and follicular fluid samples (n=30) were collected from premenopausal regularly cycling women undergoing surgery for uterine leiomyomata (n=11) and adenomyosis (n=6). The median age of the subjects was 39·4±2·8 (s.d.) years old. These samples were obtained from women on days 7–13 (proliferative and preovulatory phases) of the menstrual cycle. The diameter of each follicle was measured and follicular fluid was collected and stored at −20°C until use. The follicles were characterized according to their size, i.e. small (<5 mm; n=11), medium (5–10 mm; n=10) or large (>10 mm; n=9). Immediately following aspiration of follicular fluid, the ovaries were placed in phosphate-buffered saline (PBS) containing 10% formalin. Formalin-fixed ovaries were embedded in paraffin. All women gave their informed agreement for participation in this study which was approved by the Hamamatsu University Hospital Ethical Committee.

Cell preparation and culture

Human granulosa-lutein cells were obtained by follicular aspiration from regularly menstruating women (n=20) undergoing oocyte retrieval for in vitro fertilization because of either tubal obstruction or infertility of the spouse as described previously (Sakata et al. 2000). The cells were resuspended in culture medium (McCoy’s medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM l-glutamine), cell viability was assessed by trypan blue exclusion. The average viability was ~90%. Cell number varied from 1·5×10^6 to 7×10^6 cells per patient. The cells were finally plated into 12-multiwell plates (Costar, Cambridge, MA, USA) at a density of 2×10^5 cell per 2 ml well and incubated at 37°C in a humidified, 95% air–5% CO2 incubator for 2 days, since granulosa cells obtained from oocyte retrieval have already been exposed to gonadotropins for many hours. Culture medium was then aspirated, cells were rinsed with PBS and fresh medium was added in the presence of saline or human chorionic gonadotropin (hCG) preparation (30 ng/ml, containing a mixture of 0·1 U/ml follicle-stimulating hormone (FSH) and 0·1 U/ml hCG) for the periods of time indicated (up to 8 h). Cell extracts and culture medium were used for determination of LP concentrations by Western blotting and enzyme-linked immunosorbent assay (see below).

Extraction and isolation of cartilage LP

Proteoglycans and other soluble matrix constituents were extracted from the cells in 4 M guanidium chloride (Hascall & Sajdera 1969) and 50 mM sodium acetate buffer, pH 6·0, containing protease inhibitors (Oemega et al. 1975) for 48 h at 4°C.

In experiment 1, the extracts were dialyzed overnight at 4°C against 0·5 M guanidium chloride, 50 mM sodium acetate buffer, pH 6·0, containing protease inhibitors (Oemega et al. 1975). The extracts were directly subjected to CsCl density gradient centrifugation under associative conditions as described previously (Roughley et al. 1982). After gradient centrifugation, the fractions of density >1·41 g/ml were pooled (sample 1). This pool would be expected to contain proteoglycan molecules, HA and any proteins that associate with HA or proteoglycans.

In experiment 2, the filtered extracts were mixed at 4°C with bovine cartilage HA for 2 h, then dialyzed to associative conditions using 50 mM sodium acetate buffer, pH 6·0, containing protease inhibitors and subjected to associative CsCl density gradient centrifugation (Roughley et al. 1982, Bolton et al. 1999) (sample 2). The addition of HA ensured complete aggregation of human ovary-derived cartilage LP and proteoglycans and also enabled the majority of these proteins to be recovered in the high-density fractions of the gradients.

Purification of the LP

Cartilage LPs were isolated from proteoglycan aggregates by dissociative CsCl density gradient centrifugation,
followed by gel filtration. In brief, proteoglycan preparations were chromatographed under associative conditions on a 1 × 25 cm column, packed with Sepharose CL-2B (Pharmacia Biotech AB, Uppsala, Sweden) and run at a flow rate of 7.5 ml/h at 4 °C to remove non-aggregated proteoglycan molecules. Proteoglycan aggregates elute close to the void volume of the column (Roberts & Pare 1991). The proteoglycan aggregates in the pooled fractions were dissociated by resuspension in 4 M guanidium chloride. Chromatography on Sepharose CL-4B in 4 M guanidium chloride, 50 mM sodium acetate buffer, pH 6.0, was then used in an attempt to separate the cartilage LPs. The cartilage LP fractions (Donohue et al. 1988) were pooled, concentrated and stored at −20 °C (sample 3).

Preparation of polyclonal antibodies

Cartilage LP was prepared from bovine nasal cartilage as described previously (Kobayashi et al. 1998, 2000). Antisera obtained from immunized rabbits were specific for cartilage LP. Characterization of anti-cartilage LP antibody has been reported (Kobayashi et al. 1998, 2000). Proteins of the ITI family were detected by rabbit antibody against human ITI (Dako, Copenhagen, Denmark).

Immunohistochemistry for cartilage LP and HA

Paraffin-embedded mounted sections of human ovaries were incubated with the polyclonal antibodies raised against cartilage LP (1:100 dilution) in PBS containing 2% bovine serum albumin for 16 h at 4 °C, and then incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (1:200 dilution; Dako) for 2 h in the dark. Control sections were processed in parallel by replacement of the primary antibodies with rabbit IgG or buffer alone or the primary antibodies preincubated with an excess of purified cartilage LP, and no detectable staining was seen.

Human granulosa-lutein cells were seeded into Lab-Tek culture slides (Nunc, Roskilde, Denmark) at a density of ~10⁴ cells/chamber in the complete medium supplemented with and without 30 ng/ml hCG preparation in duplicate and cultured in a humidified atmosphere of 5% CO₂ in air. To confirm colocalization of HA-supplemented with and without 30 ng/ml hCG preparations were chromatographed under associative conditions were pooled, concentrated and stored at −20 °C (sample 3).

Enzyme-linked immunoaffinity assay for cartilage LP

Ninety six-well microtiter plates were coated with 50 µl (100 µg/ml) HA conjugated to fluoresceinamine (HA–FA; a gift from Seikagaku Kogyo Co.) in PBS at 4 °C overnight. HA–FA has a high affinity for plastic while HA does not. For studies of specificity, the same amount of chondroitin sulfate–FA or heparan sulfate–FA (a gift from Seikagaku Kogyo Co.) as HA–FA was used. One hundred microliters of the sample or cartilage LP standard were placed into each well overnight at 4 °C. The plates were incubated with biotinylated anti-cartilage LP antibodies (2 µg/ml; 100 µl/well), followed by 100 µl of avidin–peroxidase (Dako; 1:4000 in Tris-buffered saline–Tween, 20/0.5 M NaCl). Absorbance was measured at 450 nm on a microtiter plate reader.

SDS-PAGE and Western blot

Follicular fluid, culture medium, LP preparations (samples 1 and 2) or purified LP obtained from bovine nasal cartilage and human ovary (sample 3) were analyzed by SDS-PAGE (4–20% gradient gel) under non-reducing and reducing conditions with polyacrylamide gel, followed by electrophoretic transfer to polyvinylidene difluoride (PVDF) membranes as described previously (Towbin et al. 1979, Roberts & Pare 1991). The PVDF membrane was immunoblotted with anti-cartilage LP antibody (1:2000 dilution). Bands were visualized with the enhanced chemiluminescence (ECL) detection system (Amersham, Tokyo, Japan). The membranes were then placed between two transparencies and exposed to Fujicolor film. In all experiments, some strips were incubated with non-immune rabbit (or mouse) IgG as a negative control. The possibility exists that some matrix proteins (samples 1 and 2) may be covalently linked to the proteoglycans and/or HA; such molecules would remain at the top of the separation gel and would not be visualized by this technique. Therefore, these samples were treated with or without 100 U/ml Streptomyces hyaluronidase.

Statistical analysis

All statistical analysis was performed using StatView for Macintosh. Results are presented as means ± s.d. Statistical analyses were done using ANOVA. The Mann–Whitney U test was used for comparisons between the values in different groups. P<0.05 was considered significant.

Results

The level of cartilage LP in human follicular fluid

The primary focus of the present study was changes in follicular cartilage LP levels during follicle development of
The expression of cartilage LP in the follicular fluid of small and large follicles was examined by Western blot analysis using polyclonal antibodies raised against purified cartilage LP. There were multiple immunoreactive cartilage LP bands. As shown in Fig. 1, four species of immunoreactive cartilage LP of 120, 48, 42 and 15 kDa were detected in each follicular fluid under non-reducing conditions (lanes 1–4). However, the slowest migrating, immunoreactive 120 kDa faint band was not detected under reducing conditions (lane 5). Therefore, it was presumed to represent the fully glycosylated mature form of LP complexed with other proteoglycans or it may be a dimer/trimer of the fully glycosylated species. The 48 kDa protein was consistent with the fully glycosylated mature form of cartilage LP. It is therefore possible that the 42 kDa species is either a partially glycosylated form of cartilage LP or a partially degraded fully glycosylated mature form of cartilage LP (see Discussion). Whatever their identity, they represent a small proportion of the total immunoreactive material. The intensity of these bands varied significantly with respect to follicle size. Western blot analysis revealed that cartilage LP was significantly increased in follicular fluid of the large size follicle (lane 4). No protein bands were detected in the samples when the primary antibody was omitted from the Western blot protocol (data not shown).

Immunohistochemical localization of cartilage LP in human ovary

Ovaries from 17 women were examined for the localization of cartilage LP by immunohistochemistry. We employed immunostaining for cartilage LP in ovaries obtained at different times during the menstrual cycle. Cartilage LP was constitutively positive in theca-interstitial and stromal cells of any stage of follicles. Very little positive staining for cartilage LP was found in the granulosa layer of primordial, primary or secondary follicles. As shown in Fig. 2A, cartilage LP was selectively expressed in the granulosa layer of the preovulatory dominant follicle; it was detected predominantly in the extracellular matrix of cumulus cells and weakly in the mural granulosa cells. Cartilage LP was also found in the oocyte and cytoplasm of corpus lutein cells. Therefore, the expression of cartilage LP in the granulosa cells varied significantly with respect to follicle development. In a separate experiment, we found that the cartilage LP expression pattern showed no relation to the menstrual cycle (data not shown). Specific immunostaining was not observed when the antibodies were pretreated with an excess of purified cartilage LP. Immunohistochemical staining with non-immune IgG also showed no signal. Thus, cartilage LP was specifically expressed in granulosa-lutein cells, corpus lutein cells and in oocytes. On the other hand, HA was negligible in theca-interstitial cells of any stage of follicles. HA was also selectively localized in the granulosa layer of the preovulatory dominant follicle (Fig. 2B). These results demonstrated that HA-positive cells were labeled with cartilage LP specifically in the preovulatory dominant follicle. However, some cells with LP-positive cartilage (for example, oocyte and corpus lutein cells) showed no or very faint staining for HA.

Cartilage LP expression in cultured granulosa-lutein cells determined by enzyme-linked immunoadsorbent assay, immunoblotting and immunocytochemistry

We assessed the possibility of cartilage LP expression in human granulosa-lutein cells in culture. The release of
cartilage LP by granulosa-lutein cells was assessed by enzyme-linked immunoaffinity assay (Fig. 3). Cells were first treated with 30 ng/ml hCG preparation for the periods of time indicated in medium containing 10% FBS (Fig. 3A). Although unstimulated cells showed constitutively detectable levels of cartilage LP release into the medium, treatment of the cells with 30 ng/ml hCG preparation did not result in a statistically significant increase in the cartilage LP release. However, treatment of the cells with 30 ng/ml hCG preparation resulted in a significant increase in the cartilage LP content in cell extracts towards the end of culture. Time-course studies indicated that gonadotropin induced cartilage LP expression 2 h after hCG addition and the effect was sustained for up to 8 h. We observed the concentration dependence of the induction of cartilage LP levels by the hCG preparation for 8 h. As shown in Fig. 3B, maximal responses were observed with 30–100 ng/ml hCG preparation. These results indicated that the increase in cartilage LP production may be linked with the gonadotropin-stimulation pathway and cartilage LP produced by the cells is not released into the medium within an 8-h incubation.

Immunoblotting was undertaken in an attempt to determine the approximate molecular size of the LP immunoreactive substances found in the cell extract. Representative Western blots from two different sets of cells treated with or without hCG preparation (30 ng/ml) are shown in Fig. 4A. Analysis of 50 µg of the protein from the cell extract identified double bands at 48 and 42 kDa, indicating the presence of cartilage LP in the human granulosa-lutein cells. These bands correspond immuno-logically to cartilage LP of similar size in the bovine nasal cartilage-derived LP. In the gonadotropin-stimulated cells, a minor band at 15 kDa was also recognized by the anti-cartilage LP antibody. Quantitation of the levels of 48 kDa cartilage LP species on the Western blots by densitometry is shown by the numbers under each lane. The units of measurement are densitometric units, and the data are expressed as the mean of two separate analyses. Culture of cells with 30 ng/ml hCG preparation for 2 h induced a twofold increase in cartilage LP concentrations in cell extracts compared with cells cultured in media supplemented without gonadotropin. Time-course studies of cartilage LP modulation by gonadotropin revealed that cartilage LP concentration in the cell extract markedly increased after an 8-h incubation with a three- to fivefold induction relative to unstimulated cells. Gonadotropin-stimulated cumulus cells had significantly higher cartilage LP levels than the non-stimulated cells in a dose-dependent manner (Fig. 4B), which was also confirmed by the enzyme-linked immunoaffinity assay (Fig. 3) and immunohistochemical data (Fig. 5).

In a parallel experiment, media conditioned by cultured cells were subjected to immunoblotting for cartilage LP. As shown in Fig. 4C, the 48 kDa species generated by cells was very faintly immunoblotted by the anti-cartilage LP antibodies employed. The 120 kDa band seemed to be constitutively expressed in culture medium. Thus, our data demonstrated that the stimulation was time- and dose-dependent and that activation by gonadotropin induced production of cartilage LP. Of note, however,
gonadotropin-stimulated cumulus cells did not stimulate release of cartilage LP into the medium. Even under reducing conditions, there were no significant differences in the electrophoretic mobilities of the 48, 42 and 15 kDa LP species (Fig. 4A, lane 5; Fig. 4B, lane 7; Fig. 4C, lane 4). The 120 kDa immunoreactive LP is presumed to represent the fully glycosylated mature form of LP (48 kDa) complexed with other proteoglycans, since, under reducing conditions, the 120 kDa LP species was not detected (Fig. 4C, lane 4).

Immunofluorescence staining for HA and cartilage LP in human granulosa-lutein cells in culture

Figure 5 shows representative immunofluorescence staining for HA (Fig. 5A) and cartilage LP (Fig. 5B) in human granulosa-lutein cells in culture. Freshly isolated granulosa-lutein cells were first treated for 48 h in the complete medium containing 10% FBS and then incubated with the addition of 30 ng/ml hCG preparation for up to 8 h. Both HA and cartilage LP were detected predominantly on the extracellular matrix and in the cytoplasmic area, but not in the nucleus. Dual immunofluorescence staining (Fig. 5C) showed that most of HA-positive cells were found to be a cartilage LP-positive subpopulation 8 h after addition of hCG preparation. HA- or cartilage LP-like immunoreactivity was eliminated when the primary ligand or antibody was preincubated with an excess of HABP or cartilage LP respectively (data not shown).

Analysis of macroaggregates in the granulosa-lutein cell extracts

HA-rich matrix (samples 1 and 2) was extracted from the cells treated with gonadotropin. The acquisition of aggregates composed of HA was investigated using conventional SDS-PAGE. Figure 6 shows that, when macroaggregates in the cell extracts were analyzed, most immunoreactivities of HA (lane 1), ITI (lane 2), and cartilage LP (lane 3) were considered to be recovered as high molecular mass complexes that did not enter the gel (Fig. 6A). When the macromolecules were treated with Streptomyces hyaluronidase, proteins of the ITI family and cartilage LP were dissociated from the high molecular weight complexes and then migrated within the gel (Fig. 6B). These results suggest the presence of the macroaggregates composed of the HA–ITI–cartilage LP in the granulosa-lutein cell extracts. The intensities of specific bands observed in sample 2 were stronger than those in sample 1, implying that the cell extracts obtained from sample 1 contain proteins of the ITI family and cartilage LP that are able to bind HA. Finally, cartilage LP was isolated from proteoglycan aggregates by dissociative CsCl density-gradient centrifugation, followed by gel filtration (Fig. 6, A

Figure 3 (A) Time-course and (B) concentration-dependent effect of hCG preparation on the release and production of cartilage LP in human granulosa-lutein cells in culture. (A) Cells cultured for 2 days in medium containing 10% FBS were washed and then treated without (open symbols) or with (solid symbols) 30 ng/ml hCG preparation for 2 h and 8 h. The LP concentration was analyzed in the culture medium (squares; ng/ml) and cell extract (circles; ng/mg protein). (B) For concentration-dependence studies, cells were treated for 8 h with increasing concentrations of hCG preparation (3, 10, 30 or 100 ng/ml). *P<0·05 compared with control levels.
sample 3). Analysis of the isolated protein from the cell extract identified the 48 kDa main band and 42 kDa species, indicating the presence of cartilage LP in the human granulosa-lutein cell extracts.

**Discussion**

The present study demonstrates for the first time the pattern of distribution, expression and function of cartilage LP in human ovary and in cultured granulosa-lutein cells. Here we showed (1) that expression of cartilage LP is selectively detected in the granulosa cell compartment of the preovulatory dominant follicle, as well as in the corpus lutein cells and in the oocyte (Fig. 2); (2) that cartilage LP was detected by a specific and functional enzyme-linked immunoassay (Fig. 3) and Western blot (Figs 1, 4 and 6): cartilage LP was accumulated in gonadotropin-stimulated granulosa-lutein cell extract (Figs 3, 4 and 6), rather than in the culture medium in a dose- and time-dependent manner; (3) that co-localization of cartilage LP and HA was immunocytochemically demonstrated (Figs 2 and 5); and (4) that the formation of HA–ITI–cartilage LP complexes on the extracellular matrix of gonadotropin-stimulated granulosa-lutein cells was clearly demonstrated (Fig. 6). Thus, cartilage LP is thought to stabilize the aggregates by binding to both HA and ITI. The present data on cartilage LP expression extend the rodent studies to human cells.

There was significant variation in expression with respect to the size of the follicle. Thus, the size of the follicle exerts influence on the LP expression, with expression highest in larger follicles just before ovulation. Although the molecular mechanism responsible for cell differentiation and the regulation of cartilage LP expression by gonadotropin are unknown, gonadotropins and/or sex steroids may play an important role in the regulation of ovarian differentiation (Andreu et al. 1997). It has been reported (Peng et al. 1991, van Tol et al. 1996) that after stimulation with pregnant mare’s serum gonadotropin, expression of hCG (luteinizing hormone (LH)) receptor mRNA was enhanced in granulosa cells of large tertiary follicles and hCG (LH) receptor mRNA levels were dramatically decreased, particularly in the granulosa-lutein cells of preovulatory follicles, to reach the lowest levels just before ovulation. We found, however, that hCG preparation induces the expression of cartilage LP in vitro in cumulus cells when LH receptors are not present in
these cells. The hCG preparation used in the current study contains LH and FSH at a molar ratio of approximately 1:1; 30 ng/ml hCG preparation contains 0.1 U LH/ml and 0.1 U FSH/ml. Therefore, we have speculated that FSH present in the hCG preparation may directly or indirectly stimulate the process on cartilage LP expression in the cultured granulosa-lutein cells. However, at this time, we cannot explain the molecular mechanism by which cartilage LP expression is significantly induced by FSH. Since gonadotropin regulates the production of several ovarian growth factors, it is likely that some growth factor such as insulin-like growth factors may stimulate cartilage LP production (Curtis et al. 1992).

The material cross-reactive with antibodies against cartilage LP is present as several molecular species of 120, 48, 42 and 15 kDa. The major cartilage LP species present in the extracellular matrix of human granulosa cells was of 48 kDa and to a lesser degree 42 kDa. This pattern is similar to that observed in human cartilage, in which it has been shown that there are three different molecular mass LP species (48, 44 and 41 kDa), that the 48 and 44 kDa molecules differ from one another in glycosylation in a domain close to the NH₂ terminus of the protein and the 41 kDa molecule differs from the 48 and 44 kDa molecules in that it lacks a peptide of 16 amino acids, which bears these two potential glycosylation sites, close to the NH₂ terminus (Poole et al. 1984, Vijayagopal et al. 1985, Nguyen et al. 1989, Neame & Barry 1994). It is therefore possible that the 48 kDa species is described as the fully glycosylated mature form of cartilage LP and that the 42 kDa species reported in this work is either a partially glycosylated form of cartilage LP (44 kDa) or a partially degraded fully glycosylated form of cartilage LP (41 kDa). The differences in glycosylation of cartilage LP may occur as the result of under-glycosylation. Although the 15 kDa is different from the molecular mass reported for the species of cartilage LP, this species is considered to be a proteolytic product of cartilage LP. The slowest migrating, immunoreactive 120 kDa band, which might correspond to serum-derived LP (Fig. 5C), is presumed to represent the mature glycosylated form of LP complexed with other proteoglycans. Another possibility is that it may be a dimer/trimer of the fully glycosylated species that is often observed in such electrophoresis gels.

Recent experiments with generated targeted mutations in mice in the gene encoding LP (Crtl1) demonstrated that

![Figure 5](image-url)
homozygotes showed defects in cartilage development and delayed bone formation with short limbs and craniofacial anomalies (Watanabe & Yamada 1999). These results suggest that cartilage LP is important for the formation of proteoglycan aggregates in cartilage. Until now, however, there are no published data as to whether the LP knockout mouse exhibits ovarian dysfunction. In addition, the knockout mouse model, by targeting the gene of the light chain of ITI (bikunin), demonstrated that the cumulus oophorus had a defect in forming the extracellular HA-rich matrix during expansion, leading to severe female infertility (Sato et al. 2001, Zhuo et al. 2001). The bikunin is considered to be one of the binding proteins for cartilage LP (Kobayashi et al. 2000). Intraperitoneal administration of ITI fully rescued the defects (Zhuo et al. 2001). Therefore, the animal model revealed that binding of ITI to HA is important to make up the HA-rich matrix aggregate. Our unpublished data showed that exogenous cartilage LP might serve, in part, to significantly enhance the process of COC expansion possibly by stabilizing the HA–ITI (or heavy chains) complex on the surrounding cumulus cell matrices. Taken together, cartilage LP, an extracellular matrix protein in cumulus cells, stabilizes aggregates of ITI and HA, giving a cumulus cell HA-rich matrix.

We carried out these experiments to define the role of cartilage LP in the cumulus cell–oocyte communication using granulosa-lutein cells obtained at oocyte retrieval for in vitro fertilization. Granulosa-lutein cells were incubated for 2 days in the absence of exogenous gonadotropin, since these cells had already been exposed to exogenous gonadotropins for many hours. We cannot rule out the possibility, however, that these experiments, in reality, examined cartilage LP expression in early corpus luteum. Notwithstanding these limitations, this is the first immunohistochemical, immunologic and biochemical demonstration of cells specifically expressing cartilage LP in human ovary and to determine how the expression of cartilage LP is regulated. An interesting observation in this study is that human luteinized granulosa cells frequently produce cartilage LP and form the HA-rich matrix composed of, at least, HA, ITI and cartilage LP in response to gonadotropin. Our results support the hypothesis that cartilage LP may provide meaningful regulatory input in the course of human COC expansion. The presence of cartilage LP predominantly on the extracellular matrix of the luteinized granulosa cells or cumulus cells suggests that cartilage LP is localized to the HA-rich matrix where an efficient trap and stabilization of HA and ITI would take place immediately after it is released from the cells. We presume that the cartilage LP expression on the granulosa-lutein cells is mediated by some product made by the cells in response to the gonadotropin. This introduces one important issue for further study: what is the factor(s) that mediate(s) cartilage LP expression in response to the gonadotropin? The in situ hybridization technique and Northern blot analyses are also necessary to detect cartilage LP transcript in granulosa-lutein cells and/or in oocytes. Further studies

**Figure 6** Analysis of macroaggregates in the granulosa-lutein cell extracts. Macromolecules obtained from the cell extracts (samples 1, 2, and 3) as described in Materials and Methods were treated without (A) or with (B) Streptomyces hyaluronidase and analyzed by SDS-PAGE followed by western blotting with biotin–HABP (for detection of HA; lane 1), anti-ITI (lane 2) or anti-cartilage LP (lane 3) respectively. The right-hand panel (sample 3) shows the results of SDS-PAGE (left lane) and western blot using anti-cartilage LP antibody (right lane). The asterisk indicates the top of the separation gel; the 75 and 90 kDa double bands, heavy chains of the ITI family; the 48 kDa double bands, the fully glycosylated mature cartilage LP and possibly its degradation product during experimental procedure; and the 25 kDa band, deglycosylated bikunin (light chain of ITI). The 42 kDa band could not be detected in this sample 2 (B, lane 3), but repeated experiments revealed the presence of a faint band of the 42 kDa species (data not shown). Molecular weight markers are shown on the left.
are required to elucidate the biological actions of cartilage LP in the ovary.

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