Covalent Linkage between Proteins of the Inter-α-inhibitor Family and Hyaluronic Acid Is Mediated by a Factor Produced by Granulosa Cells*

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The direct interaction of hyaluronic acid (HA) and proteins of the inter-α-inhibitor family plays a critical role in organization and stabilization of the expanding cumulus extracellular matrix (cECM) following an ovulatory stimulus. Despite similarities in the morphology of cumulus oocyte complexes (COCs) expanding in vivo and in vitro, we find that the cECM of COCs which expand within intact follicles are more elastic and resistant to shear stress than the cECM of those stabilized in vitro. Western blot analysis shows that only the heavy chains of inter-α-inhibitor are incorporated into the cECM and appears to be covalently linked to HA after stabilization in vivo while intact inter-α-inhibitor is bound to the HA-enriched cECM by a non-covalent mechanism in in vitro stabilized COCs. However, purified pre-α-inhibitor and HA can form covalent linkage in the presence of granulosa cells or with granulosa cell-conditioned medium. In addition, COCs resistance to shear stress is also enhanced by coinoculation with granulosa cells. Upon formation of the apparent covalent linkage between heavy chains and HA in culture medium, the light chain (bikunin) is concomitantly released into the medium as a complex with chondroitin sulfate moieties of inter-α-inhibitor supporting the possibility that HA may replace the chondroitin sulfate linkage to the heavy chains. We speculate that a factor(s) secreted by granulosa cells within the follicle may catalyze a transesterification reaction resulting in an exchange of chondroitin sulfate with HA at the heavy chain/chondroitin sulfate junction followed by release of chondroitin sulfate-bikunin into the follicular fluid. It is also possible that the subsequent further stabilization of the cECM through the covalent interaction of HA and heavy chains of inter-α-inhibitor may play an important role in the process of ovulation.

In most mammalian species (including mouse, rat, and human), cumulus-oocyte complexes (COCs) of pre-ovulatory follicles undergo a dramatic change following an ovulatory stimulus. The tightly packed cumulus cells first disaggregate and then synthesize and secrete large amounts of hyaluronic acid (HA) into their extracellular matrices (ECMs). The ECM, cumulus cells, and oocyte are thus integrally bound within an expanded mucoid complex which is about 20 to 40 times larger (volume) dependent upon the species (1). This process of cumulus expansion is required for ovulation and may also facilitate the process of fertilization (2–4).

We have previously identified a serum factor (proteins of the inter-α-inhibitor family), critical in organizing and stabilizing the expanding cumulus matrix (5). This protein factor appears to be excluded from follicular fluid until the ovulatory gonadotropin surge and then quickly diffuses into the follicular fluid where it becomes integrated within the cumulus ECM (5, 6). Two major forms of this factor, pre-α-inhibitor (PαI) and inter-α-inhibitor (IαI), exist in mammalian species including mouse, bovine, and human (7, 8). They each include a common light chain (about 40 kDa) which has two domains of the Kunitz-type trypsin inhibitor and so this protein is termed bikunin. PαI is composed of bikunin and a single heavy chain connected by chondroitin sulfate (9–12). IαI consists of bikunin and two heavy chains also joined by chondroitin sulfate. According to a model proposed by Enghild et al. (10, 11), a single chondroitin sulfate chain extends from a glycosylation site at Ser-10 of the bikunin subunit to link with the C-terminal Asp residue of each heavy chain via an ester bond to form a novel carbohydrate linkage. The three different heavy chains are highly homologous and, in fact, the specific heavy chain combinations identified in different species may differ from one another. For example, PαI of human and mouse is composed of heavy chain 3 (HC3) and the light chain, while bovine PαI consists of heavy 2 (HC2) and the light chain (13).

Both PαI and IαI are almost identical in their ability to stabilize the expanding cumulus ECM in vitro (14) where a direct interaction between proteins of the IαI family and HA seems to play a critical role in preventing the release of HA into the culture medium. As demonstrated in an earlier in vitro study, this initial interaction appears to be a non-covalent charge-mediated interaction (14). Although COCs which expand in vitro are morphologically indistinguishable from those expanding in vivo, ovulated COCs appear to be more elastic and more resistant to mechanical shear force. It has been reported that proteins of the IαI family could form covalent interactions with HA in various systems including follicular fluid (15–18), however, the degree of native protein that forms covalent linkage with HA appears to be very low and the mechanism of the covalent interaction has not been clarified. Nonetheless, it is possible that a covalent interaction between...
I and HA could result in this observed increased stability of the cumulus ECM.

In this study, we show that the majority of I and HA within the ovulated cumulus ECM is covalently linked with HA and that this covalent interaction can be partially achieved in vitro by incubating purified Pol and HA with granulosa cells. Like ovulated COCs which expand within the intact follicle, COCs stabilized in medium containing granulosa cells or granulosa cell-conditioned medium, possess greater resistance to shear forces than those stabilized in medium lacking granulosa cells or granulosa cell-conditioned medium. This increased stability may be required for maintenance of integrity of the cumulus mass during extrusion of the COC through the rupture site within the follicular wall.

**EXPERIMENTAL PROCEDURES**

**Materials**

Female mice (B6D2F1; 18–22 days old) were purchased from Harlan (Indianapolis, IN). Minimal essential medium (MEM) with Earle’s salts were obtained from Life Technologies, Inc. (Grand Island, NY). Fetal bovine serum (FBS) was from Hyclone (Logan, UT), and pregnant mare’s serum gonadotropin was purchased from Diosynth (Chicago, IL). Rabbit anti-human IgG (1 mg/ml) was obtained from Dako (Carpinteria, CA). Carrier-free [35S]sulfuric acid was from ICN (Irvine, CA). Chondroitinase ABC was from Boehringer Mannheim and porcine chondroitinase antibiotic, human choricongonadotropin (hCG), glucosamine, rooster comb hyaluronic acid, Streptomyces hyaluronidase, guanidine-HCl, and lauryl sulfobetaine were all purchased from Sigma.

**Preparation of COCs—**Mice were injected with 5 IU of pregnant mare’s serum gonadotropin and sacrificed 48 h later. Ovaries were placed in MEM with penicillin-G (100 units/ml) and streptomycin (50 μg/ml). COCs (about 50–80 COCs per animal) for in vitro expansion assays were isolated and incubated in medium containing MEM, 2.5 mM glucosamine, porcine follicle-stimulating hormone (2 μg/ml), and lauryl sulfobetaine were all purchased from Sigma.

**Methods**

Preparation of COCs—Mice were injected with 5 IU of pregnant mare’s serum gonadotropin and sacrificed 48 h later. Ovaries were placed in MEM with penicillin-G (100 units/ml) and streptomycin (50 μg/ml). COCs (about 50–80 COCs per animal) for in vitro expansion assays were isolated and incubated in medium containing MEM, 2.5 mM glucosamine, porcine follicle-stimulating hormone (2 μg/ml), and other factors (FBS, purified bovine or mouse Pol as specified in each experiment) at 37 °C and 5% CO2 for 16 h as described previously (5). In vivo stabilized ovulated COCs were collected about 12 h after an injection of an ovulatory dose of hCG (5 IU) in animals primed 48 h earlier with pregnant mare’s serum gonadotropin (5 IU).

High Performance Liquid Chromatography Coupled ELISA for COCs—Ovulated COCs were washed 3 times in phosphate-buffered saline and then transferred to 500 µl of 6 mM guanidine HCl with 8% lauryl sulfobetaine or to 100 µl of phosphate-buffered saline with 2 units of Streptomyces hyaluronidase for 3 h at 37 °C and then transferred to 400 µl of 6 mM guanidine HCl with 8% lauryl sulfobetaine. About 100 µl of each of these samples were fractionated using an SV40 large T antigen transgenic mouse provided by Dr. J. S. Butel, Baylor College of Medicine. The cells were maintained and propagated in MEM supplemented with 10% FBS at 37 °C under 5% CO2. After achieving confluence in a 75 cm2 flask, the cells were washed 3 times with phosphate-buffered saline and then incubated with 5 ml of labeling medium (sulfate free MEM containing 2 mM carrier-free [35S]sulfuric acid, 0.5% FBS) overnight. The medium was centrifuged (1000 x g) for 10 min and passed through a 0.2 µm filter (Millipore) to remove cell debris. The incorporated radioactivity was removed using ultracentrifugation with a molecular mass cut-off of 100 kDa (Centricone-100, Amicon) and the medium concentrated to a final volume of 0.5 ml. About 20 µl of this labeled protein mixture was then added to 80 µl of MEM containing granulosa cells under various conditions specified in the figure legends and incubated overnight at 37 °C and 5% CO2. These cell-medium mixtures were then centrifuged (at 1000 x g for 10 min) to remove cell debris and 2 µl of anti-rabbit human I IgG conjugated goat anti-rabbit IgG (1:1000), the blots were developed using substrates according to the manufacturer’s instructions (Bio- Rad).

Preparation of Chondroitin Sulfate Radiolabeled I and Immunoprecipitation—To radiolabel the chondroitin sulfate component of I, a mouse hepatoma cell line was generated from an SV40 large T antigen transgenic mouse provided by Dr. J. S. Butel, Baylor College of Medicine. The cells were maintained and propagated in MEM supplemented with 10% FBS at 37 °C under 5% CO2. After achieving confluence in a 75 cm2 flask, the cells were washed 3 times with phosphate-buffered saline and then incubated with 5 ml of labeling medium (sulfate free MEM containing 2 mM carrier-free [35S]sulfuric acid, 0.5% FBS) overnight. The medium was centrifuged (1000 x g) for 10 min and passed through a 0.2 µm filter (Millipore) to remove cell debris. The incorporated radioactivity was removed using ultracentrifugation with a molecular mass cut-off of 100 kDa (Centricone-100, Amicon) and the medium concentrated to a final volume of 0.5 ml. About 20 µl of this labeled protein mixture was then added to 80 µl of MEM containing granulosa cells under various conditions specified in the figure legends and incubated overnight at 37 °C and 5% CO2. These cell-medium mixtures were then centrifuged (at 1000 x g for 10 min) to remove cell debris and 2 µl of anti-rabbit human I IgG conjugated goat anti-rabbit IgG (1:1000), the blots were developed using substrates according to the manufacturer’s instructions (Bio- Rad).

RESULTS AND DISCUSSION

In vitro and in vivo expanded COCs exhibit marked differences in resistance to shear forces. In vivo stabilized, ovulated COCs exhibit shear resistant indices greater than 60 in every ovulated COC tested (n = 12). In fact, trituration of ovulated COCs was arbitrarily terminated at the 60th cycle since every ovulated COC tested in this manner was still intact. In sharp contrast, in vitro stabilized COCs (stabilized in the absence of granulosa cells) exhibit a shear resistance index of 8 ± 2 (n = 26), while those stabilized in vitro in the presence of granulosa cells exhibit a shear resistance index of 20 ± 4 (n = 23). While the number of cumulus cells in either conditions were not assessed, incubating COCs with granulosa cell-conditioned medium also resulted in an enhancement of the shear resistance
granulosa cells is non-covalent in nature. In contrast, major Iα components of in vivo stabilized COCs could not enter the gel upon treatment of the sample with SDS and 2-mercaptoethanol (lane 6) without prior hyaluronidase treatment. The Coomassie Blue staining of the transferred gel shows similar transfer efficiency in both lanes 6 and 7 (not shown). After Streptomyces hyaluronidase treatment prior to SDS and 2-mercaptoethanol of the in vivo stabilized COCs, however, prominent immunostaining was visible with a major component of Iα at about 100 kDa (probably the heavy chain) and a minor component migrating at about 200 kDa (probably a double heavy chain; see Fig. 3, below). There is, however, a very small amount of immunopositive material corresponding to the heavy chains and the native Pα in the sample that is not treated with hyaluronidase (lane 6, compare to lane 7). It may be that a small amount of heavy chain spontaneously falls off during extraction. If all of the immunopositive material shown in lane 7, following hyaluronidase treatment, is covalently linked with HA, the conversion from native protein to the covalently bound form is almost complete. Such a high degree of covalent linkage between heavy chains of the Iα family and HA in an extracellular matrix is unprecedented.

The time course of incorporation of Iα heavy chains into the expanding HA enriched cumulus ECM in vivo is shown in Fig. 3. During the time frame from 3 to 12 h after the hCG injection, only trace amounts of intact Iα/Pα became incorporated into the ECM as shown by Western blot (lanes 2–4) without prior treatment with hyaluronidase. However, a large amount of Pα/Iα heavy chains were incorporated into the matrix by about 6 h after injection of hCG revealed by treating the sample with Streptomyces hyaluronidase (lanes 5–7). There is no detectable incorporation of native protein or heavy chains of Pα/Iα during the first hour following hCG, regardless of whether the samples are treated with hyaluronidase or not (not shown). In addition, a band was again observed at about 200 kDa, which was suspected to be comprised of double heavy chains, possibly derived from the Iα that forms a covalent linkage with a single HA molecule in such a way that it is protected from the action of hyaluronidase. Indeed, further treatment of the hyaluronidase-treated sample with NaOH (0.1 M for 10 min at room temperature followed by neutralization with 0.1 M HCl; a method previously shown to dissociate the heavy chains from O-linked carbohydrates as well as the ester bond that links the heavy chain of Iα with chondroitin sulfate (9, 18)), the 200-kDa band disappeared and only the single

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**Fig. 1. HPLC coupled ELISA of Iα of ovulated COCs.** Protein samples were fractionated on a TSK-G-4000 gel filtration column running at a 6 ml/min flow rate. Fractions were collected from 10 to 34 min which includes the void volume (~1000 kDa) to 10 kDa. 100 µl of each fraction were used to coat polywall plates and ELISAs were performed using anti-human Iα IgG as described under "Experimental Procedures." A, 20 µg of purified bovine Pα. B, 20 µg of purified bovine Pα treated with 0.1 unit of chondroitinase ABC. C, 157 ovulated COCs solubilized in water containing 6 µM guanidine HCl and 8% lauryl sulfobetaine. D, 192 ovulated COCs treated with 1 unit of Streptomyces hyaluronidase for 3 h before solubilization in water containing 4.8 µM guanidine HCl and 6.4% of lauryl sulfobetaine.

**Fig. 2. Western blots of Iα of in vivo ovulated COCs and in vitro stabilized COCs.** Protein samples were treated with SDS and 2-mercaptoethanol before loading on the gel as described in detail under "Experimental Procedures." Lane 1, high molecular weight prestained standards. Lane 2, 1 µl of mouse serum. Lane 3, 5 µg of purified mouse Pα treated with 0.5 unit of chondroitinase ABC showing the location of heavy chains and the light chain. Lane 4, equivalent of 20 COCs stabilized in 10% mouse serum. Lane 5, 20 COCs stabilized in 10% mouse serum and treated with 1 unit of hyaluronidase. Lane 6, 10 in vivo ovulated COCs. Lane 7, 10 in vivo ovulated COCs treated with 1 unit of Streptomyces hyaluronidase.
apparent heavy chain was present (lane 10). In addition, this 200-kDa band is not sensitive to consecutive treatments with hyaluronidase and chondroitinase (lane 14). Moreover, direct treatment of ovulated COCs with NaOH (without pretreatment with hyaluronidase, lane 15) also coverts this 200-kDa band to a sharp 100-kDa band that corresponds to the position of the heavy chain of IαI. These results taken together support the possibility that a small fraction of the two heavy chains of IαI form a covalent linkage with the same HA molecule in close proximity that is resistant to Streptomyces hyaluronidase or chondroitinase but sensitive to NaOH treatment. The lanes between 7 and 8 as well as lane 11 are the high molecular weight standards.

The apparent covalent interaction observed in vivo within the ovulated cECM can be partially reproduced in vitro in the presence of granulosa cells or granulosa cell-conditioned medium. This system consisted of HA, purified IαI or Pol, and granulosa cells in MEM or granulosa cell-conditioned medium. As shown in Fig. 4A, only when granulosa cells or granulosa cell-conditioned medium are added into the reaction mixture, will the system generate the free light chain of Pol and the heavy chain of Pol which is released upon treating the sample with Streptomyces hyaluronidase (Fig. 4A, lanes 6–9). Heat-treated granulosa cell-conditioned medium is unable to facilitate the covalent binding of HA with heavy chain (Fig. 4A, lanes 4 and 5). The same experiments illustrated in lanes 6–9 of Fig. 4A were repeated and illustrated in lanes 2–5 of Fig. 4B but with a higher concentration of purified Pol and HA. As stated above, Western blot of medium extracts showed two bands corresponding to Pol and bikunin (Fig. 4B, lanes 2 and 4). Treatment of the samples with Streptomyces hyaluronidase prior to SDS and 2-mercaptoethanol treatment, however, revealed a prominent band corresponding to the heavy chain position (Fig. 4B, lanes 3 and 5). Purified bovine Pol displayed the same pattern of interaction with HA when incubated with granulosa cells or granulosa cell-conditioned medium (not shown). The identity of the 50-kDa band as free bikunin (light chain) of Pol was strengthened by using bikunin site-specific antiserum in the Western blot. In this experiment, a sample of purified Pol was electrophoresed before (Fig. 4B, lanes 7 and 9) or after treatment with chondroitinase ABC to dissociate the light and heavy chains (Fig. 4B, lanes 8 and 10). The commercial anti-human IαI rabbit IgG recognizes native protein (lane 7) and the dissociated heavy chain and light chain (lane 8). In contrast, the bikunin site-specific antiserum only recognizes native Pol (lane 9) and the light chain (lane 10). Lane 12 is a Western blot illustrating an experiment in which the anti-bikunin site-specific antiserum was used to detect the presence of bikunin-positive epitopes after a sample of Pol was incubated with granulosa cells and HA and then subsequently treated with Streptomyces hyaluronidase. As expected, the Western blot only showed the native protein band and the light chain. No heavy chain was detected. It should noted that the migration patterns for IαI/Pol and bikunin are somewhat different in different Western blots in this figure because different percentages of polyacrylamide were utilized as specified in the figure legend.

After treatment of native Pol with chondroitinase ABC, the bikunin (light chain) fraction always appeared as two closely migrating bands on Western blot (e.g., Fig. 2 and Fig. 4B). This pattern may reflect the heterogeneity in the length of chon-
dextran sulfate linkage in the native protein which has been shown to vary from 16 to 21 polysaccharide units (11, 12). Alternatively, it is possible that the unit of polysaccharide that connects the heavy chain and light chain may not be exclusively composed by chondroitin sulfate such that alternative cutting sites of chondroitinase may exist on the polysaccharide. This later possibility is consistent with the present study showing that upon forming the covalent linkage with HA, the released light chain-chondroitin sulfate complex migrates as a single band in Western blots (Fig. 4).

The amount of heavy chain binding covalently in our in vitro system is also dependent upon the concentration of exogenous HA (Fig. 5). The amount of native protein apparently binding covalently with HA progressively increases with increasing exogenous HA as judged by the progressive loss of native protein bands (lanes 3–6). In contrast, incubation of purified Pol and HA with granulosa cells alone (Fig. 5, lane 2) or with exogenous HA alone in MEM (Fig. 5, lanes 7–9), does not generate any detectable heavy chain covalently bound to HA and the intensity of the native protein band is unaltered.

The physiological significance of the covalent interaction of the heavy chains of Pol/I with HA is not yet clear. However, further stabilization of the cumulus ECM seems to be achieved by this interaction as quantified indirectly by the shear-resistance assay in vitro. It should be pointed out that the efficiency of Pol/I incorporation into the ECM in vitro is much lower than that in vivo (Fig. 2, compare lanes 5 and 7). Since this low level of Pol/I incorporation occurs even at high serum concentration (10%), we are currently unable to adequately assess the status of the Pol-HA complex in COCs stabilized in vitro when coincubated with granulosa cells. It is unlikely that the moderate enhancement of shear resistance of COCs by coincubating with granulosa cells results from recruitment of granulosa cells into the expanding COCs because the addition of granulosa cell-conditioned medium results in almost the same degree of enhancement. However, Salustri et al. (22) have shown that ovulated COCs have about 3 times more cumulus cells than those compact COCs expanded in vitro and that the origin of those extra cumulus cells occurs by recruitment of mural granulosa cells. Thus, the high shear resistance of in vivo stabilized COCs may occur as a consequence of recruitment of granulosa cells by the inner layer of the cumulus. Since granulosa cells possess the ability to catalyze the covalent interaction between heavy chains of Pol/I and HA, this may also lead to the packing and incorporation of large amounts of heavy chain into the expanding ECM. We speculate that the additional stabilization possibly achieved through the covalent interaction of heavy chains of Pol/I and HA, may provide elasticity required by the complex to maintain its integrity and to protect the oocyte during its extrusion from the ruptured follicle.

The current study also supports the likelihood that only the heavy chain of Pol/I and Pol forms a covalent linkage with HA while bikunin is concomitantly released into the culture medium when they are incubated with granulosa cells or in granulosa cell-conditioned medium. It is plausible to speculate that this process may be catalyzed or assisted by a factor(s) secreted by granulosa cells in response to an ovulatory stimulus. We have postulated that this conversion may involve an enzyme (esterase) synthesized by granulosa cells which catalyzes a transesterification between HA and chondroitin sulfate at the junction between the carboxyl end of the heavy chain of Pol/I and chondroitin sulfate where chondroitin sulfate serves as the linker between the heavy chain and light chain (23). Indeed, Huang et al. (16) originally proposed that an enzyme(s) in serum could catalyze the exchange of HA with the chondroitin sulfate moiety of Pol/I based upon the observation that incuba
tion of serum with HA generates a heavy chain that is covalently linked with HA. In our system, however, overnight incubation of either purified Ia/Pa or serum (both mouse and bovine) with various amounts of HA alone could not generate any detectable heavy chain-HA complex or free bikunin. While the proportion of heavy chains covalently binding with HA after incubation in serum was not reported (16), conversion from a charge-mediated interaction between Pa/Ia and HA to a covalent binding of the heavy chain and HA in vivo matured COCs is virtually complete. Serum may, however, contain a very low level of the hypothetical enzyme capable of catalyzing this charge mediated to covalent conversion. It will be interesting to determine whether or not long-term incubation of purified Ia and HA can spontaneously generate a low level of heavy chain-HA complexes.

More recently, Zhao et al. (24) have found that Ia heavy chain-HA complexes isolated from pathological synovial fluid form an ester bond with the C-terminal Asp residues of Ia heavy chain. This finding and the present study are consistent with the transesterification model of covalent binding of the heavy chain and HA involving exchange of HA and the chondroitin sulfate component of Ia as depicted in Fig. 6. This model predicts that formation of the covalent linkage with the Ia heavy chain results in release of the chondroitin sulfate moiety of the native molecule along with bikunin. This was partially confirmed by radiolabeling the chondroitin sulfate moiety of the native molecule along with bikunin. This was further experimentally confirmed by communication with A. Salustri concerning the mechanism of the HC-HA covalent interaction are also greatly appreciated.

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