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Localization and Thyroid Hormone Influenced Expression of Collagen II in Ovarian Tissue

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Key Words

Collagen II • Ovary • Ovarian Granulosa cells • Hypothyroid • Matrix metalloproteinase

Abstract

Collagen type II (Col II), one of the main components of the hyaline cartilage, is a member of the fibril-forming collagen family. Due to its amino acid composition, the extent of lysine hydroxylation of Col II is much higher than that of other fibril forming collagens. Since lysyl hydroxylase isoforms are less synthesized in hypothyroid ovarian tissue, Col II level is expected to be reduced here and contribute to the degradation of ovarian ECM in this condition. As there was no previous report, we have demonstrated Col II expression in rat ovary. Col2A1 mRNA shares significant part of the total collagens in ovary as shown by the relative expression of the major collagen genes present in this tissue. It has also been shown that Col II is down regulated in hypothyroid ovarian tissue and its expression is increased upon stimulation by thyroid hormone (T_3). To know whether less Col II in hypothyroid ovarian tissue is due to less synthesis of the protein or its increased rate of degradation is also involved in it, we demonstrated the status of Collagen - degrading Matrix Metalloproteinases in this condition and found up regulation of MMP-1, -8 and -13 in hypothyroid rat ovary. The present study shows the reduced Col II expression in hypothyroid rat ovary, with the concomitant increase in Col II degradation. This information will be useful for further studies on reproductive disorders.

Introduction

The most abundant proteins in the ECM are members of the collagen family, which are the major structural elements of all connective tissues. The network-forming capacity and anchoring function of certain collagen types could contribute to the formation of scaffolds, promoting tissue repair or regeneration [1-2]. The functional and structural integrity of cartilage tissue is believed to rely mainly on collagen II (Col II) molecules within its matrix [3-4]. Col II acts in concert with other collagen types [5] and matrix proteoglycans [6-7]. Col II has been localized in articular cartilage of many species including human [8-9] and its mRNA produced by chondrocytes has also been detected *in vivo* in mouse- [10] and in human cartilages [11].

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Ovary is a very dynamic tissue where tissue remodeling occurs continuously. Collagen I, -III, -IV and fibronectin are shown as main components of ovary. Ovarian granulosa cells cultured under anchorage-independent conditions produced a basal lamina containing type IV collagen [12] and fibronectin [13]. The role of T_3 in collagen expression has already been reported. The results of these studies suggest that the granulosa cells are capable of secreting many of the components of a basal lamina and T_3 influences the expression of these ECM components [14-15]. Hypothyroidism impairs reproductive functions in human being and experimental animals, although the mechanism of this dysfunction is not known. These reproductive disorders include irregular oestrous cycle, ovarian atrophy, disturbed folliculogenesis, delaying onset of puberty, anovulation, amenorrhoea or hypermenorrhoea, menstrual irregularity and increased risk of continual abortion [16-19]. Growth and ovulation of follicles, and development, maintenance, and regression of the corpus luteum depend on cyclical remodeling of the ECM. Therefore, ECM is directly or indirectly involved in many of these disorders and collagens are the major components of ECM. The ECM consists of proteinaceous and non-proteinaceous components and provides the tissue-specific, extracellular architecture to which cells attach, and modulates the activities of cells through cell surface receptors. Enzymes like lysyl hydroxylases, prolyl hydroxylases play vital role in synthesis of collagen, the main component of ECM. However continuous synthesis and degradation of collagen is necessary for the normal ovarian function and perfect balance of these two events is maintained in normal condition. Matrix metalloproteinases (MMPs) are the key enzymes to degrade these ECM components [20, 21]. MMP-1, -8 and -13 are the enzymes, which specifically break the collagens. The key feature of these enzymes is their ability to cleave interstitial collagens I, II, and III at a specific site. Collagenases can also digest a number of other ECM and non-ECM molecules. As they are collagen-specific protease, we have selected MMP-1, -8 and -13 for our study.

The percentage of lysine hydroxylation of Col II is much higher compared to other collagen types [22]. As lysyl hydroxylase isoforms are less synthesized in hypothyroid ovary, we thought it would be important to investigate the status of Col II in hypothyroid condition, as there will be more chance of Col II getting affected by less lysyl hydroxylase synthesis in ovary than other collagen types. Therefore, considering the enormous possible importance of Col II in performing ovarian function in normal as well as in hypothyroid condition, we started this investigation.

In this study we were interested to see whether Col II is present in ovarian tissue and at the same time its status in hypothyroid condition. Our findings confirm that Col II is expressed in ovary and its expression is regulated by T_3 and collagen-degrading enzymes (MMP-1, -8 and -13) are up regulated in hypothyroid condition. Hypothyroidism, therefore, reduces Col II synthesis with concomitant increase in its degradation in the rat ovary.

Materials and Methods

Animals and treatment

Pregnant Sprague Dawley rats raised in our animal facilities were housed in a well ventilated and temperature controlled room with a 12h light and 12h darkness schedule. They were fed with standard balanced rat pellet and drinking water was made available ad libitum. Rats were divided into two groups, 1) euthyroid, were provided with normal drinking water and their pups were used as control. 2) hypothyroid, mother rats were administered 0.02% 6-N-propyl-2-thiouracil (PTU, Sigma, St. Louis, MO) dissolved in drinking water until the end of experiment and their pups were rendered hypothyroid as they take the drug through mother's milk [23]. Hormone treatment consisted of daily single ip injections of 15ng T_3 (Sigma) per gm body weight. At 28 days of age, the total ovary or ovarian granulosa cells were isolated and were pooled to isolate RNA. 10 pups from each group were pooled in each experiment. At the end of the treatment periods, animals were euthanised under ether anesthesia. The Institutional Animal Ethics Committee approved all animal protocols that were followed during the experiments.

Granulosa cell isolation

The ovarian granulosa cells were isolated from 28-day old female pups. The cells were obtained from the ovaries by puncturing the follicles with fine (26 gauge) needles gently allowing expulsion of cells into 1X PBS (ice cold). Pooled cells were collected by brief centrifugation, washed and were resuspended in RPMI medium and then kept in a humidified atmosphere containing 5% CO_2, 95% air at 37°C. The cells were cultured for four hours when the effect of T_3 was examined in *in vitro* condition, one hour without T_3 and three more hours after addition of T_3 in the culture medium. The cell viability was more than 90% in all sets of experiments, as measured by the trypan blue dye exclusion test.

Radio Immuno Assay (RIA)

For determination of plasma thyroid hormone levels, 100µl blood from rats was collected and quickly mixed with 100µl ice-cold 0.9% NaCl containing 0.24mg EDTA. Plasma was separated by cold centrifugation and the samples were stored at -80°C until the assays were performed. Plasma T_3 was determined by RIA using T_3 RIA kit (Riak-4, Board of Radiation and Isotope

Technology, Bhaba Atomic Research Center, Mumbai, India). After incubation, the tubes were thoroughly decanted and the bound radioactivity was determined by a γ-counter (Electronics Corporation of India Limited, India). Standard curves were constructed by plotting the amount of total radioactivity bound against the hormone concentration. The sensitivity of T_3 was 0.24ng/ml of the sample based on 90% B/B_0 intercept.

ELISA

ELISA was performed for serum TSH using Pathozyme TSH kit (Omega Diagnostics Ltd., Alva, UK) following manufacturer's instructions. In short, the samples, standards and enzyme conjugate were dispensed into antibody coated well, incubated 1hr at RT (20-25°C) and washed. To this, substrate Tetramethyl benzidine (TMB) was added and incubated in the dark for 20min for colour development. The absorbance was noted immediately in plate reader (Qualigens, Mumbai, India) using 450nm primary filter. The inter assay CV was 6% and intra assay CV was less than 5%. The minimum detectable concentration of TSH by Pathozyme TSH kit was estimated to be 0.2μIU/ml.

Immunohistochemistry

The ovaries were dissected out and fixed in 10% paraformaldehyde, 5μm thick paraffin embedded ovarian sections were deparaffinized by dipping into xylene for 20min. The sections were dehydrated by passing through graded alcohol, blocked in 10% pre-immune serum and was processed for immunostaining. Anti-collagen II goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, dil 1:100) was added as primary antibody and incubated for 2hs, washed and then incubated with secondary antibody (rabbit anti-goat AP, Santa Cruz, dil 1:100) for 1h. Collagen II blocking peptide (Santa Cruz, dil 1:10) was reacted with anti-collagen II antibody and used for staining specificity. Primary antibody was omitted in negative control section. Immunoreactions with NBT/BCIP were visualized under the Zeiss Axiovert 25 microscope, (Carl Zeiss, Gottinger, Germany).

Immunofluorescence microscopy

Rat ovarian granulosa cells were fixed on cover glass (BD Bio-coat, USA) with 1 X PBS containing 4% PFA for 2h at 4°C. The cells were permeabilized with 0.1% TritonX-100 in 1X PBS. The cells were then incubated with goat polyclonal anti-collagen II antibody (Santa Cruz, USA, dil 1:50) for 2h followed by incubation with FITC-conjugated secondary antibody (Donkey anti-goat, Santa Cruz, USA, dil 1:100) for another 1h, with rigorous washing in between all these steps using 1XPBS. 1μg/ml DAPI was also added to each sets. The stained cells were observed under a Fluorescence microscope (Olympus BX51 microscope, Tokyo, Japan) and the images were captured with cool snap pro camera.

Western blot analysis

Total ovaries from 10 individual rats were isolated for each group, i.e. for control, hypothyroid and T_3-treated hypothyroid groups, for each experiment. The ovaries, thus pooled, were homogenized in homogenizing buffer (150mM NaCl, 500mM Tris and 10mM EDTA) supplemented with protease inhibitors (1μg/ml aprotinin, 1μg/ml pepstatin, 1μg/ml leupeptin, 1mM phenylmethylsulfonyl fluoride, and 1μg/ml trypsin inhibitor) and 1% Triton X-100 (all from Sigma chemical co, USA). The homogenate was then centrifuged at 8000g for 10min at 4°C. The supernatant was collected and (an aliquot of it was used for protein concentration estimation) resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA). The membrane was incubated with 5% blocking solution (Trisbuffered saline containing 0.1% Tween 20, and 5% non fat dried milk) for 1h, washed twice with Tris-buffered saline containing 0.1% Tween 20 and then incubated for 16h with goat anti-collagen II, anti- MMP-8, anti-MMP-13 or mouse anti-MMP-1 respectively (1:1000 dilution in 5% blocking solution) and mouse anti-actin antibody was used for loading control (1:2000 dilution in 5% blocking solution, all from Santa Cruz Biotechnology, USA). Immunoreactive bands were visualized by reaction of horseradish peroxidase (HRP)-labeled secondary donkey anti-goat or rabbit anti-mouse antisera at 1:2000 dilutions with HRP substrate [24]. Bands were quantified by calculating area and pixel value data (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/). Each band was measured by integrated density, and after background correction of the image, corrected pixel density was taken.

RNA isolation and cDNA preparation

Total RNA was isolated from the ovary as well as from ovarian granulosa cells using TRI Reagent (Sigma) following the manufacturer's instruction and the method described earlier [25] and cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (MBI fermentas, Hanover, MD) following the manufacturer's instruction.

Sequencing and analysis

Sequencing of the PCR products were performed by ABI Prism Automatic DNA Sequencer (Perkin Elmer, Wellesley, MA). Sequence alignment and data analysis was performed through BLAST search from NCBI GenBank and using ClustalW software.

Reverse Transcription-Polymerase Chain Reaction

2μl of the cDNA, prepared by RevertAid M-Mulv Reverse Transcriptase (MBI Fermentas) was used as template for RT-PCR with gene specific primers and relative expression was observed with GAPDH primer. A 50μl PCR volume was made by adding 2.5U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), to a PCR mixture containing 1X reaction buffer [50mM KCL, 10mM Tris-HCl (pH 8.3), 0.1% Triton-X-100 and 2.5mM $MgCl_2$], 200μM of each dNTPs (MBI Fermentas), 20pmol of each primers. The PCR was performed for 25 cycles, each cycle consisting of denaturation at 94°C for 30s (5min in the first cycle), annealing at specific temperature for each set of primers for 30s, extension at 72°C for 30s (10min in the last cycle; Perkin-Elmer 9700). The RT-PCR product was cloned and sequenced. The oligonucleotide primers used in the reactions have been listed in Table 2.

Real Time quantitative PCR

Relative Quantitative RT-PCR (Q-PCR) was performed on iCycler real time PCR machine (Bio-Rad Laboratories, Hercules, CA) using DyNAmo SYBR Green Q-PCR Kit (Finnzyme, Finland) following the instructions provided by the vendor to confirm the changes in gene expression observed during semi-quantitative RT-PCR. An internal control GAPDH gene was amplified in separate tubes in each experiment [16]. The oligonucleotide primers used in the reactions have been listed in Table 3.

C_T value calculation

In Q-PCR, relative quantification was performed by comparative C_T value calculation. In this method arithmetic formulas are used to calculate relative expression levels, compared with a calibrator, which can for instance be a control (non-treated) sample. The amount of target normalized to an endogenous housekeeping gene and relative to the calibrator, is then given by $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = \Delta C_T$ (sample) $- \Delta C_T$ (calibrator), and ΔC_T is the C_T of target gene subtracted from the C_T of the housekeeping gene [26].

Statistical analysis

All data are expressed as the mean ± SD, and statistical analysis were performed using Student's t-test. $P<0.05$ was considered to be significant. Experiments were repeated at least three times in duplicate unless otherwise stated. To make the variance independent of the mean, statistical analysis of the Q-PCR data was performed after logarithmic transformation.

Results

Col2A1 gene expresses in ovarian tissue

To find out whether col2A1 gene is expressed in ovarian tissues, RT-PCR was performed with the ovarian RNA using the gene-specific primers as mentioned in the Table 2. The PCR product was then electrophoresed on agarose gel and the specific band was obtained (Fig. 1). The amplified product was cloned and sequenced and the sequencing data shows that this was indeed col2A1 gene as confirmed by homology search of the published col2A1 gene sequence (GenBank Acc. No L48440).

Immunolocalisation of collagen II in rat ovary

To localize Col II, rat ovarian sections were prepared and immunohistochemistry was performed using collagen II antibody. The immunostaining data suggests that collagen II protein is expressed both in theca and in granulosa cells (Fig. 2). The staining was also observed in extra cellular matrix. No immunostaining was observed in absence of primary antibody (negative control) in this tissue.

	T_3 (ng/ml)	TSH (μIU/ml)
Con	2.6 ± 0.03	0.32 ± 0.032
Con+PTU-T_3	0.495 ± 0.025	3.75 ± 0.021
Con+PTU+T_3	2.2 ± 0.01	0.22 ± 0.015
Con-PTU+T_3	3.65 ± 0.008	0.25 ± 0.011

Table 1. Serum T_3 and TSH levels in control and in experimental rats. Serum samples were collected from the control, hypothyroid, T_3-treated hypothyroid and T_3-treated control rats and serum T_3 (by RIA) and TSH (by ELISA) were assayed as mentioned in the methods section.

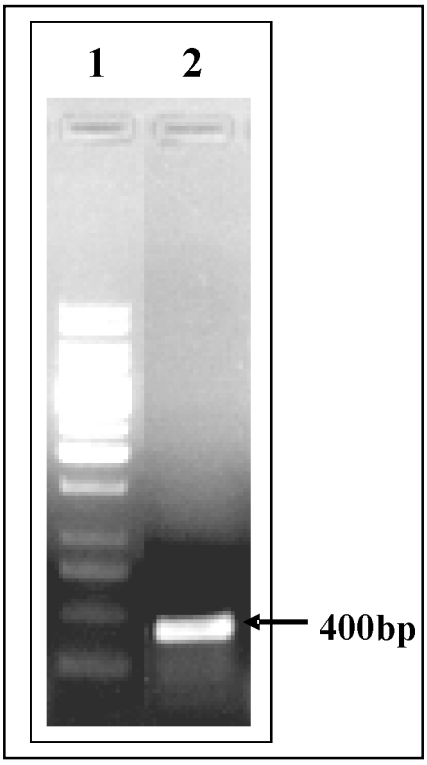

Fig. 1. Expression of col2A1 gene in ovary. RT-PCR was performed using ovarian RNA with col2A1 gene specific oligonucleotide primers. The amplified product was loaded on agarose gel (lane 2) and 1kb DNA ladder, size marker, was loaded on lane 1.

Localization of collagen II in ovarian granulosa cell

To observe the localization of Col II in the ovarian granulosa cells, fluorescence immunocytochemistry was performed with collagen II antibody followed by FITC-tagged secondary antibody (Fig. 3). The immunofluorescence microscopic data confirms the presence of Col II in the ovarian granulosa cells (a), as there was no signal in absence of primary antibody (negative control, d). The corresponding DAPI stained cells (b) confirm the presence of Col II in the cytoplasm.

Table 2. The oligonucleotide primers and the respective amplicon sizes of different genes used in the semi quantitative RT-PCR has been listed.

Gene Product	Acc.no.	Forward Primer	Reverse Primer	Size of the amplicon
GAPDH	BC059110	5'GCCATCAACGACCCCTTC3'	5'AGCCCCAGCCTTCTCCA3'	237 bp nt-164-400
Col2A1	L48440	5'ACCGAGGTTTCACTGGAC TG 3'	5'TGGTTGTTCAGCGAC TTGAG3'	400 bp nt-3332-3731

Table 3. The oligonucleotide primers and the respective amplicon sizes of the different genes used in the Q-PCR have been listed.

Gene Product	Acc.no.	Forward Primer	Reverse Primer	Size of the amplicon
GAPDH	BC059110	5'GCCATCAACGACCCC TTC3'	5'AGCCCCAGCCTTC TCCA3'	237 bp nt-164-400
MMP1	NM_002421	5'AGGGGATGCTCATTT TGATG3'	5'ACCGGACTTCATC TCTGTCG3'	449 bp nt-647-1095
MMP8	NM_022221	5'CCACAGATGTCAAAG GCTGA3'	5'AGGTTGGACAGGG TTGTCTG3'	447bp nt-367-813
MMP8	NM_022221	5'CCACAGATGTCAAAG GCTGA3'	5'AGGTTGGACAGGG TTGTCTG3'	447bp nt-367-813
Collagen I (Col1A1)	Z78279	5'TAAAGGGTCATCGTG GCTTC3'	5'ACTCTCCGCTCTT CCAGTCA3'	501bp nt-3287-3787
Collagen II (Col2A1)	L48440	5'ACCGAGGTTTCACTG GAC TG 3'	5'TGGTTGTTCAGCG ACTTGAG3'	400 bp nt-3332-3731
Collagen III (Col3A1)	XM_343563	5'GATCAGATGGTCAGC CAGGT3'	5'CATCTTTTCCAGG AGGTCCA3'	495bp nt-2997-3491
CollagenIV (Col4A3)	L47281	5'CTGCTTACAGCGATT CACCA3'	5'GTAGTTGCAGGTC CCTCGTC3'	397bp nt-156-552

Col II mRNA shares substantial part among the major collagen types in ovary

To investigate the expression level of Col II compared to other major collagen types in ovarian tissue, we isolated total RNAs from this tissue and Q-PCR was performed using the gene-specific primers of Col I, -II, III and IV (Table 3). The C_T value suggests that the expression level of Col II is very much comparable with other major collagen types in ovary (Table 4).

Table 4. The Q-PCR of different collagen genes and their respective C_T value have been listed.

Gene	C_T value
Collagen I (Col1A1)	30.1
Collagen II (Col2A1)	29.8
Collagen III (Col3A1)	28.8
CollagenIV (Col4A3)	29.3

Down regulation of Collagen II and up regulation of MMP-1, -8 and -13proteins in hypothyroid condition

For Western blot analysis the whole homogenate from the ovarian tissues was prepared and the supernatants were subjected to immunoblot analysis with the antibodies against collagen II. Immunoblotting in parallel sets were performed with actin antibody as loading controls. Col II protein level was decreased in hypothyroid rat ovary compared to control and it was increased in the ovary when the hypothyroid rats were injected with T_3, as demonstrated by Western immunoblot (Fig. 4a). The Western immunoblot data showed that the level of active collagen-specific MMPs (MMP-1, -8 and -13) was significantly increased in hypothyroid rat ovary (Fig. 4c, d and e respectively) compared to control, whereas their expression was normalized when T_3 was added back to

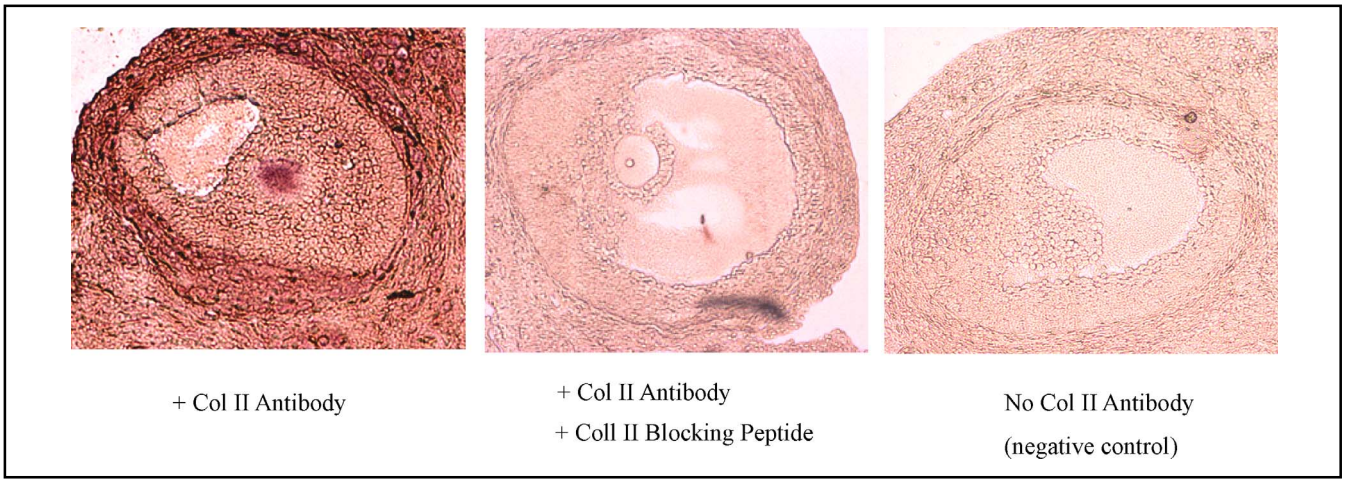

Fig. 2. Immunohistochemical localization of Col II in rat ovary. The ovarian sections were incubated with goat polyclonal anti-collagen II antibody and AP-conjugated rabbit anti-goat-IgG was used as 2° antibody. The color developed due to immunoreaction by NBT/BCIP was visualized, (X 40); whereas there was no color in peptide block section and in absence of primary antibody (negative control).

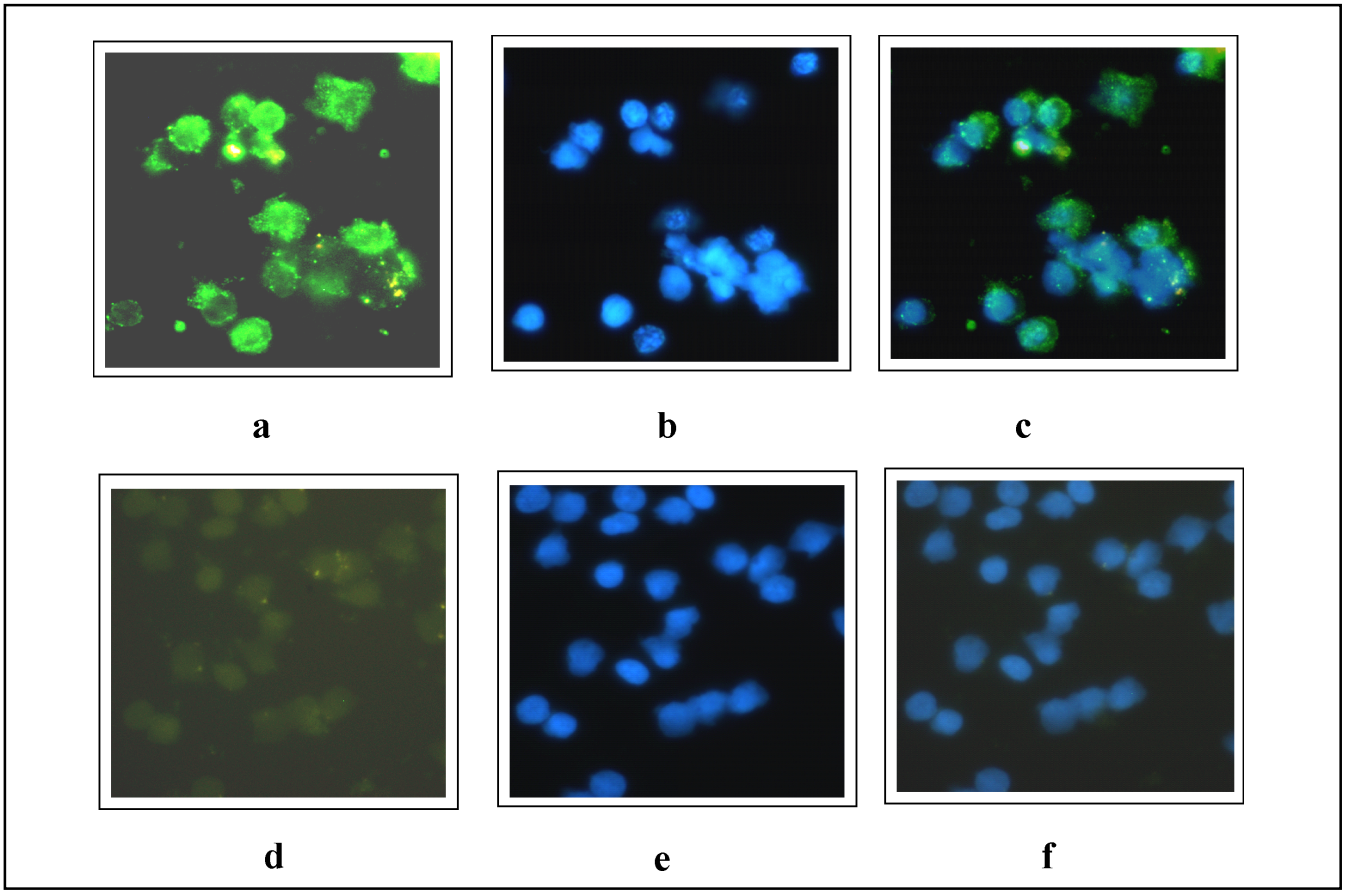

Fig. 3. Cellular distribution of Col II in ovarian granulosa cells. Immunocytochemistry of granulosa cells was performed with Col II antibody and FITC-linked secondary antibody. The cells from each set were also stained with DAPI and the cells were visualized by Olympus fluorescence microscope and photographs were captured by cool snap pro camera. The granulosa cells were treated with (a) or without (d, negative control) Col II antibody; each set were then treated with FITC-linked secondary antibody. The respective DAPI-stained cells have been shown as b and e. The experiment was performed at least 3 times in duplicates. Fig. c and f are the merged images of a (FITC) with b (DAPI) and d (FITC) with e (DAPI) respectively.

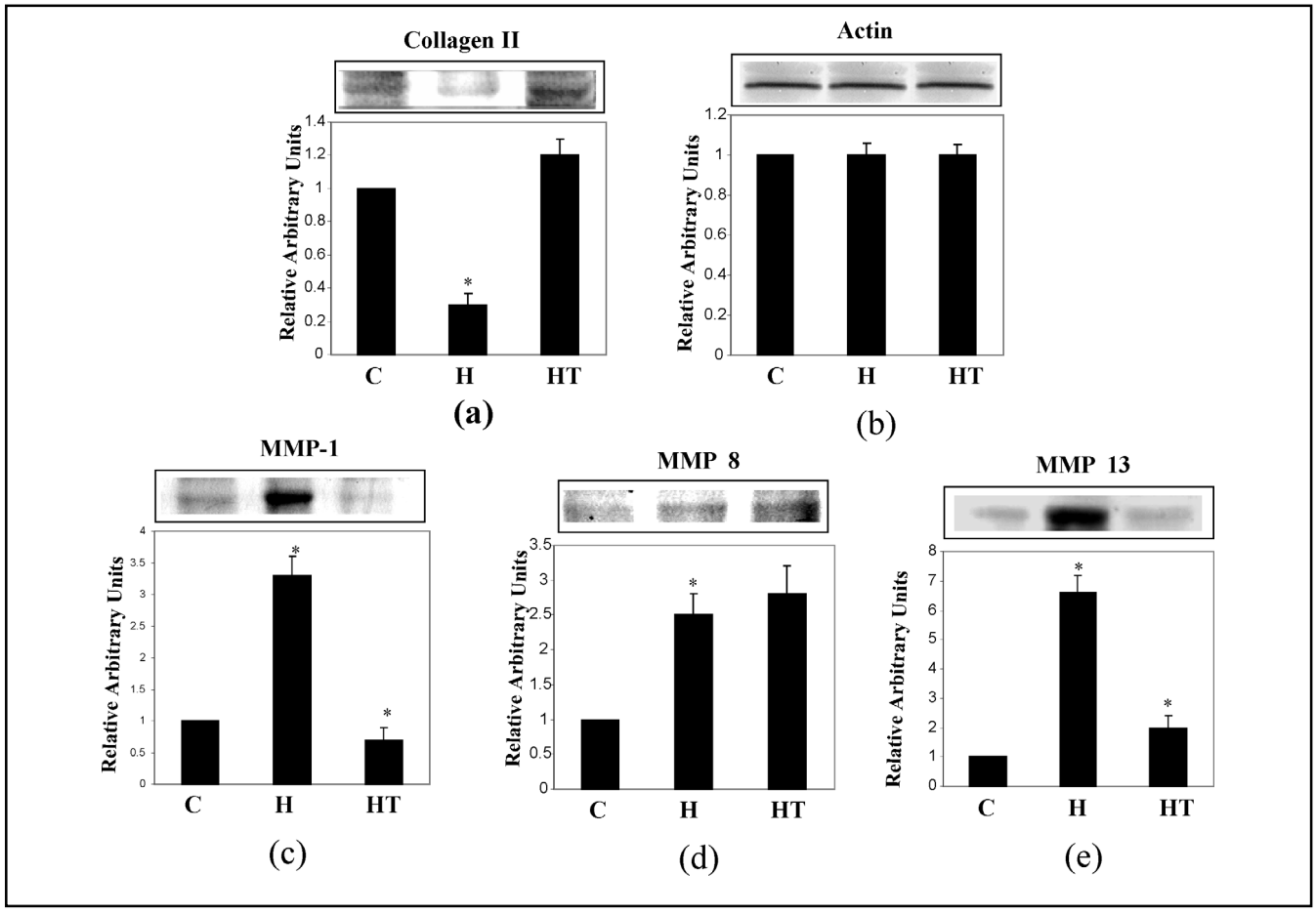

Fig. 4. Status of Col II, MMP-1, MMP-8 and MMP-13 in hypothyroid condition. 30µg of total ovarian proteins from each set were fractionated on 10% SDS-polyacrylamide gel, transferred onto PVDF membrane and subjected to immunodetection with goat anti-collagen II (a), mouse anti-MMP-1 (c), goat anti-MMP-8 (d), goat-anti-MMP-13 (e) antibodies and mouse anti actin antibody as an internal control (b). The lanes indicating 'C', 'H', and 'HT' represent the protein loaded were isolated from control, hypothyroid and T_3 injected hypothyroid animals respectively. The protein bands were quantified with ImageJ software (NIH, USA) and the lower panels of the respective figures show the pixel densities of the protein bands represented by relative arbitrary unit (RAU). The experiments were performed three times in duplicate and the mean ± SD values have been shown. *, $P<0.05$.

the hypothyroid rats. Actin antibody was used as a loading control in this experiment (Fig. 4b).

Increased expression of MMP-8 and -13 in hypothyroid condition

To elucidate the expression level of collagen degrading MMPs in hypothyroid ovary, Q-PCR was performed with ovarian RNAs isolated from control, hypothyroid and T_3-treated hypothyroid animals, using the gene specific primers (Table 3) for MMP-8 and -13. It was found that the expression of MMP-8 and -13 were significantly increased in hypothyroid ovary and their expression was reduced after addition of T_3 (Fig. 5a and b). GAPDH gene specific primers were used as internal control. The Q-PCR-amplified products were then electrophoresed on agarose gel and the band intensities show similar expression profile (c-e) as found in the Q-PCR data.

Discussion

Collagens, the major components of ECM, were once considered to be a group of proteins contributing to the extracellular scaffolding with their fibrillar structures. Collagens are essential for their binding capacity, network-

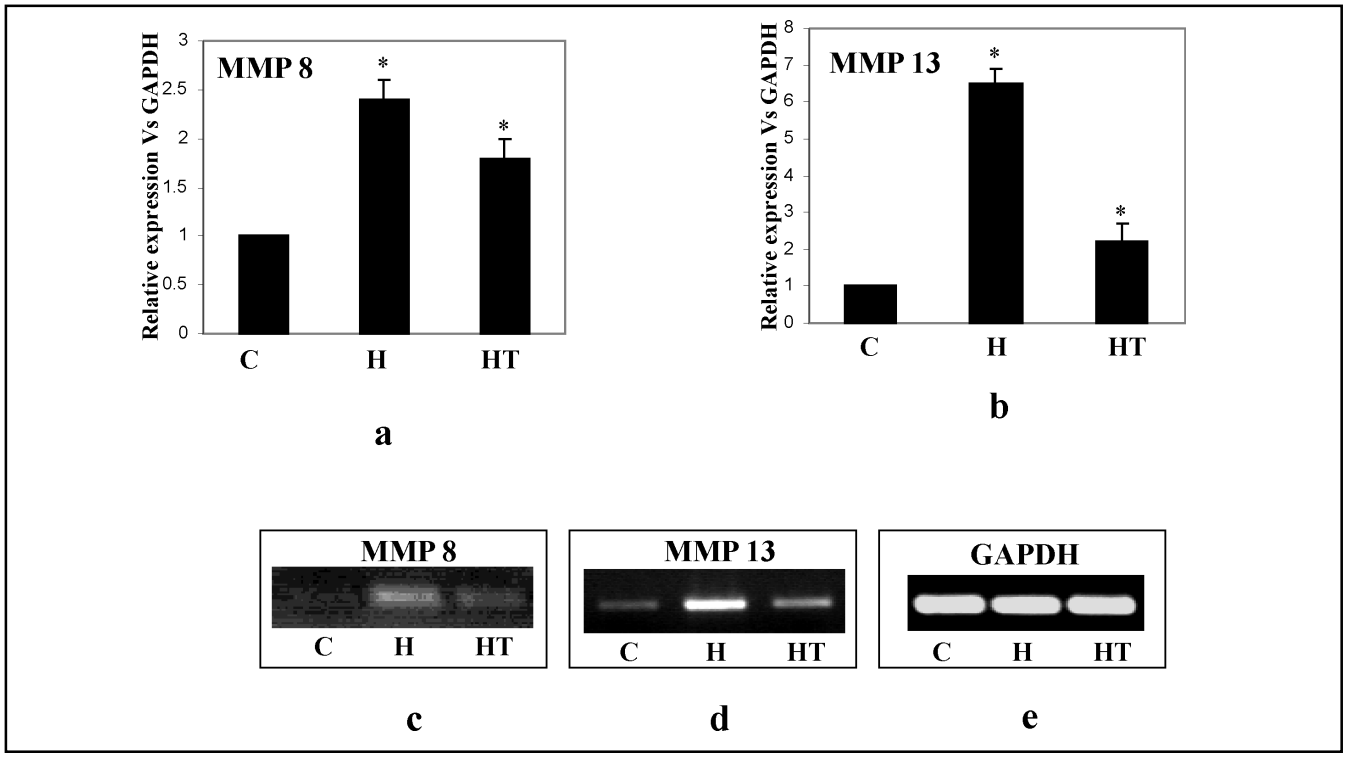

Fig. 5. Expression of MMP-8 and -13 in hypothyroid condition. The expression pattern of MMP-8 (a) and -13 (b) transcripts in control, hypothyroid and T_3-treated hypothyroid rat ovaries was measured by Q-PCR. Data are represented as fold changes in experimental condition compared to control by analyzing the C_T values corrected by those of corresponding internal GAPDH controls. Data from four experiments (mean ± SD) are determined as fold changes compared to normal rat (*, $P<0.001$). The Q-PCR-amplified products were then electrophoresed on agarose gel and ethidium bromide stained bands are shown as MMP-8 (c), MMP-13 (d) and GAPDH (e). RNA utilized for the Q-PCR was isolated from the ovaries of control (C), hypothyroid (H) and T_3-injected hypothyroid (HT) rats.

forming capacity and anchoring function. As Col II provides the rigidity and structural support to the tissues, it is expected that in ovary too, it would be an important component of the matrix. In ovary, different types of collagens are highly expressed, like collagen I, -III, -IV and others, but Col II expression has not been reported so far in this tissue.

Hypothyroidism induced reproductive malfunction is common to both the sexes although the molecular mechanism behind these disorders are still poorly known [16-19]. We have already shown the disintegrated ovarian texture due to hypothyroidism, at the same time reduced expression of procollagen lysyl hydroxylase (Plod), collagen I and -III in hypothyroid rat ovary [16]. One major difference in composition between type II collagen and other two major fibril-forming collagens, types I and III, is that the extent of lysine hydroxylation is about 2-4-fold in the former, with more than half of the hydroxylysine residues being glycosylated by galactose or glucosylgalactose [22]. Since ovary is a very dynamic tissue, tissue remodeling occurs continuously. Besides that, it is the site of follicular maturation and steroidogenesis. Therefore, maintenance of the ovarian structural integrity is inevitable and collagens, the major ECM component plays crucial role in it. We wanted to confirm the role of Col II, apart from Col I, III and IV, in maintaining the ovarian ECM in normal as well as in hypothyroid condition. Therefore, we felt necessary to confirm the presence of Col II in ovary and then to find out its role in maintaining the ovarian structure and function.

The experimental pups used in this investigation were made hypothyroid as mentioned earlier [16] and the serum T_3 and TSH of these animals were noted (Table 1). We have shown that col2A1 gene is well expressed in rat ovary as demonstrated by RT-PCR followed by sequence analysis of this PCR product (data not shown). By immunohistochemistry, Col II has been shown to localize both in theca and in granulosa cells. Apart from the follicular cells, the Col II protein has been shown to localize

in the ECM also, which indicates that after synthesis the mature Col II leaves the cells and localized in the ECM. The immunofluorescence microscopic data confirms the presence of Col II in the ovarian granulosa cell cytoplasm as shown by staining the same cells with FITC-Col II Ab and DNA-specific dye, DAPI respectively. The level of col2A1 mRNA in ovary is high enough and comparable to other major collagen types in ovarian tissue, suggesting significant contribution of Col II in the formation and maintenance of ovarian ECM.

The status of collagen in hypothyroid ovary has already been reported [16]. By western immunoblotting we showed the significant down regulation of Col II in hypothyroid state, whereas its expression was revived upon T_3 injection to the hypothyroid animals. In this report, we demonstrated by Q-PCR that the expression of collagen-specific MMPs, i.e. MMP-8 and -13 was significantly increased in hypothyroid ovary and their expression was normalized after T_3 addition. Moreover, by western immunoblotting we demonstrated that the expression of MMP-1, -8 and -13 proteins was significantly increased in hypothyroid condition and was decreased when hypothyroid animals were treated with T_3. Therefore, Col II synthesis becomes less; at the same time its degradative enzymes are also increased in hypothyroid ovary, resulting in very low amount of Col II in this tissue due to hypothyroidism. The T_3 addition experiment suggests that there is always a tendency to recover the level of Col II and the respective MMPs towards their normal level, suggesting all these effects are reversible.

Previously it was shown that collagen gene expression is influenced by T_3 receptor [14]. Hypothyroidism decreases all collagen fractions in skin and in liver and an increase of acid-soluble and insoluble collagen in bone samples [15]. Reduced lysine hydroxylation of Col II may result in low abundance of mature Col II in hypothyroidism. As Col II maturation is inhibited and the expression of collagen II-degrading MMPs is induced, it is expected that the cumulative effect of these two events would be less accumulation of Col II in hypothyroid ovarian tissue compared to control. Therefore, the reduced mature collagen formation may disturb the structural integrity of the ovarian ECM components leading to reproductive disorders. The collagen-specific MMPs, MMP-8 and -13 are probably regulated at the transcription level by thyroid hormone, hence their mRNAs and proteins both induced in hypothyroid ovary. Since rat MMP-1 gene sequence is not available in GenBank, RT-PCR using MMP-1 gene-specific primers with rat RNA was not possible. As MMP-1 antibody is available, which cross-reacts with rat, human and mouse MMP-1 as stated by the manufacturer, we performed western blotting experiment with this antibody. Like other collagen-specific MMPs, MMP-1 protein level was increased in hypothyroid ovarian tissue.

Finally, our findings show that Col II is expressed in the ovary and its expression is regulated by T_3. It is important to study Col II regulation by T_3 in the ovarian tissue as reproductive malfunction often occur due to hypothyroidism. Our work has opened the scope for such interrogation.

Abbreviations

Col II (collagen II); col2A1 (collagen II gene); ECM (extracellular matrix); MMP (matrix metalloproteinase); GAPDH (glyceraldehyde-3-phosphate dehydrogenase); TSH (thyroid stimulating hormone); PFA (paraformaldehyde); PBS (phosphate buffered saline); AP (alkaline phosphatase); DAPI (4'6-Diamidino-2-phenylindole); FITC (Fluorescin Isothiocyanate); C_T (Comparative threshold); T_3 (Thyroid hormone).

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