The connective tissue matrix of the heart remains under regulatory influence of the thyroid hormones. Some conflicting data describe the connective tissue changes in subjects with thyroid gland disorders. The aim of the study was to assess the changes of the connective tissue accumulation in the heart of rats in the state of hypothyroidism and to answer the question whether TSH is involved in mechanism of the observed phenomena. Hypothyroidism in rats was induced by methylthiouracil treatment or by thyreoidectomy. The thyroid hormones \([\text{freeT3 (fT3), freeT4 (fT4)}]\) and pituitary TSH were measured in plasma with radioimmunological method. The glycosaminoglycans (GAG) and total collagen were measured in heart muscle of both left and right ventricles. Cells from the rat’s heart were isolated and cultured. The cells were identified as myofibroblasts by electron microscopy method. The effects of TSH in concentrations ranging from 0.002 to 20 mIU/ml, on connective tissue accumulation in heart myofibroblasts cultures were tested. The primary hypothyroidism was developed both in groups with thyreoidectomy and with methylthiouracil. The levels of fT3 and fT4 both in rats with thyreoidectomy and animals treated with methylthiouracil were decreased and TSH level in these two experimental groups was elevated. In the heart of the rats with experimental hypothyroidism increased content of both GAG and collagen was found. Myofibroblast number in culture was increased by TSH. Regardless of the method of its induction, hypothyroidism increased collagen and GAG contents in the heart. TSH is not involved in regulation of collagen and glycosaminoglycans accumulation in the heart of rats affected with primary hypothyroidism.

**Key words:** collagen, connective tissue, glycosaminoglycans, methylthiouracil, myofibroblasts culture, thyroxine, thyreoidectomy, triiodothyronine, TSH

**INTRODUCTION**

Connective tissue determines the structural integrity of the heart. Cardiac fibroblast are responsible for the synthesis of fibrous proteins such as collagen or elastin, (1) and are able to release metalloproteinases, enzymes involved in collagen degradation (2). The extracellular matrix of the connective tissue provides the scaffold for the cells and determines mechanical properties as well as influence on metabolism of the heart tissue. Therefore, connections between both cardiomyocytes or myofibers and connective tissue take part in their mechanical coupling (3). Moreover, connective tissue binds the myocytes to the capillaries and helps to maintain the forward flow during systole (4). Excess accumulation of the collagen in the heart decreases its' compliance.

Glycosaminoglycans (GAG) are heteropolysaccharides consisted of the disaccharide repeating sequences, which when attached to the protein core, form proteoglycans (PG). The PG are responsible for cell attachment or migration (5). Moreover, GAG are involved in the regulation of gene expression (5, 6). Anionic groups spaced along the GAG chain, create high negative electrostatic potential that attracts small cations and repulses anions influencing the ionic balance (7). GAG may interact electrostatically with collagen and other macromolecules of the connective tissue and modify the process of collagen fibrillogenesis (7).

Several data suggest that the thyroid gland has regulatory influence on connective tissue metabolism. Triiodothyronine injections increase after 72 hours the mRNA level for both procollagen type I and III in the rat heart (8). Saha and coworkers showed reduced expression of collagen type II gene (9) and enhanced collagen degradation (10) in the hypothyroid rat ovary. On the other hand, increased staining for heparan sulphate in growth plate was seen in the hypothyroid rats (11). Triiodothyroacetic acid, the analogue of the thyroid hormone, increased of aminoterminal propeptides of human type I and type III procollagens in the cortisone-pretreated skin (12). Elevated level of GAG in the extraocular muscle and skin of the pretibial area was found in patients with the Graves disease (13). This phenomenon was evoked by serum derived factor (14). Increased urine excretion of GAG, however, reflects altered metabolism of GAG in patients with hypothyroidism (15). Thyroxin was proved to reduce high level of hyaluronic acid (HA) in the skin of patients with myxoedema (16). Moreover, increased proteoglycan synthesis was found in human fibroblasts cultured in thyroid hormones deficient medium (16).
Heart is the target organ for thyroid hormones (17). Acoustic heart structure modifications in patients with hypothyroidism were showed by echocardiography. This effect could be caused by changes of the collagen content, water retention or muscle fibers orientation in the heart (18).

Scarcely and conflicting information regarding the connective tissue metabolism in the hearts of subjects with hypothyroidism encouraged us to describe the changes of collagen and glycosaminoglycans in the heart of rats with experimental hypothyroidism. Previously described effects of the thyrotropin on peripheral tissue (19) and modification, by this hormone, of C-telopeptides of type I collagen in women (20) as well as discovery of the TSH receptors on human fibroblasts (21-23) or adipocytes (24, 25), encourage us to check, whether effects described in rats with hypothyroidism could be explained by the direct TSH influence on the cardiac myofibroblasts – the cells synthesising collagen and GAG.

**MATERIAL AND METHODS**

**Animals**

Forty male Wistar rats weighing 200 g ±30 g (±S.D.) were housed with a free access to commercial food pellets (LSM, Baculit, Poland) as well as tap water ad libitum. All the animals were kept in light (L) - dark (D) conditions L:D= 12:12. Light was turned on at 07.00. In the first series of the experiment animals were divided into four groups: Control animal - group 1 (Ct), rats with sham thyreoidectomy - group 2 (Sham-Tx), thyreoidectomized rats - group 3 (Tx) and animals treated with 4-methyl-2-thiouracil - group 4 (MTU; Fluka). 0,1% solution of MTU was applied with drinking water during two weeks. After 2 weeks all animals were decapitated and their hearts were dissected into left and right ventricles, then weight of all parts was measured. Atria were cut off and not used for analysis. Both muscle of the left and right ventricles were taken for biochemical analysis. In all samples total collagen as well as glycosaminoglycans contents were determined. On the last day of experiment, immediately after decapitation, blood was collected from the jugular vessels. The experiments were performed according to the relevant ethical law or approved by the Local Commission of Ethics.

**Thyreoidectomy**

After thiopental (Sandoz GmbH) anaesthesia (50 mg/kg b.w.), incision of the skin on the neck was made in the median line from the mandible to the sternum. Then, the sternohyoideus muscle was cut and both its left and right parts were removed sideward. Biventer muscle was separated from the thyroid gland and removed. Isthmus of the thyroid gland situated between 1st and 3rd cartilage of the trachea was cauterized with heated scalpel. The thyroid lobes were separated from the trachea and removed. Bleeding vessels were coagulated with heated scalpel. Finally, the wound was closed by four silk sutures. Sham operation was done according to the same method except the thyroid gland removal.

**Thyroid hormones evaluation**

Plasma levels of free triiodothyronine (FT3) and free thyroxin (FT4) were evaluated with commercial RIA kits produced by Immunotech. TSH concentration was measured with commercial kit specific for rat (Amersham International plc, Bucks, UK). Samples from all rats were analyzed within the same assay. The radioactivity of the samples was assessed by gamma counter (1275 Minigamma Wallac).

**Cell cultures**

The hearts of the six newborn rats (2-3 days old) were removed from the chest in the aseptic conditions and stored in RPMI1640 medium with gentamycin (25 µg/ml) and fungizone (2,5 µg/ml, Gibco). Then the hearts were minced and incubated in 0.04% collagenase solution for 30 min. (37°C, 5% CO2). After incubation, the tissue was centrifuged (5 min, 900 rpm), the supernatant was discarded and the cells were washed with DMEM (Gibco) containing 10% fetal calf serum (BioWest), gentamycin (25 µg/ml) and fungizone (2,5 µg/ml). The tissue was centrifuged (5 min, 900 rpm) and the cells were plated on dishes. After 2 hours non-adherent cells were washed out. The cells were incubated in humidified atmosphere of 5% CO2 in the air in the temperature of 37°C. The cells were grown to the confluence and then were trypsinized and passaged. To set up the new culture, the initial cells density of 8x105/cm2 was used. The number of both total cells and died cells (stained with trypan blue) were counted in the Burker chamber. Viability of the cells ranged from 87% to 96% and was not influenced by TSH. The experiments were carried out on the cells after 1st or 2nd passage. During the experiment the cells were growing in DMEM supplemented with 3% calf serum, antibiotics in concentrations given above and bovine TSH (Sigma) in concentrations: 20, 2 mIU/ml, 2 mIU/ml, 0,2 mIU/ml, 0,02 mIU/ml or 0,002 mIU/ml. The results were compared with control cultures. In the cultures the content of glycosaminoglycans and collagen was measured. Migration of the cells was assessed by wound healing assay. Briefly, the confluent cell culture was scratched by yellow tip. The width of fissure was measured by scale in ocular, until the fissure was completely covered by migrating cells.

**Electron microscopy preparation**

Cell suspensions were centrifuged in 2,5% glutaraldehyde in cacodylic buffer pH 7.4. After centrifugation (5 min, 900 rpm) pellets were washed in 0,13 M cacodylic buffer and then afterfixed in 2% OsO4 for two hours. After rinsing in 0,13 M cacodylic buffer the cells were dehydrated in increasing solutions of alcohol and additionally in propylene oxide. Specimens were embedded in synthetic resin Araldite. Thin sections, 60 nm thick, were placed on 300-mesh copper grid and contrasted with lead citrate and uranyl acetate. The cells were examined and photographed under an electron microscope Philips EM301 at the microscope magnification 5700-25000 X.

**Determination of collagen**

The collagen content was determined, with the method described by Woessner (26). For evaluation of the total collagen level, the macerated tissue was extracted with ether-acetone and vacuum-dried at 90°C. Samples of the total collagen were assayed for hydroxyproline by hydrolysis with 6N HCl (3 ml/10 mg of dry tissue) at 110°C for 24h. After hydrolysis, all the hydrolyzates were evaporated to dryness in a water bath and the precipitates were dissolved in 3 ml of distilled water. The samples, neutralized by 1N NaOH, were diluted to 10 ml with distilled water. From the tubes, 0,2 millilitre samples were taken for further analysis and diluted with distilled water to 2 ml of the final volume. Hydroxyproline was oxidized to pyrrole by 1,25 ml of chloramine T in citrate buffer (pH=6,0), then shaken for 5 min and incubated for 20 min at 20°C. In order to remove the excess of chloramine T, 1,25 ml of 3,15 M perchorlic acid was added. After 5 min, the samples were treated with 1 ml of 20% p-dimethylaminobenzaldehyde and incubated in a 60°C water bath for 20 min. The optical density was measured at 560 nm with spectrophotometer.
Determination of glycosaminoglycans

The 1, 9-dimethylmethylene blue (DMMB) assay of Farndale et al. (27) was used for the determination of GAG in the samples. Briefly, samples were homogenized, defatted with an ether-acetone mixture (3:1) and the residue was dried to constant weight at 90°C. Fifty mg of dry sample was added to the mixture composed of 0.75M NaOH and 50 mM natrium borate and incubated in water bath at 73°C for 1 hour. After incubation the pH was neutralized with 6M HCl to approximately 7.0 and then 72 µl of 100% TCA was added to each sample, to precipitate proteins. After centrifugation (6000 rpm during 30 min) 6ml of 100% ethanol was added to the supernatant. For GAG precipitation the samples were put into refrigerator (-20°C) overnight and then centrifuged (12 500 rpm during 30 min). The precipitate containing GAG was resolved in distilled water. The 1.2 ml of DMMB-reagent (Aldrich Chemical Co) was added to 50 µl of the sample and the absorbance was measured at 525 nm on a spectrophotometer. DMMB reagent is composed of 51 mM of DMMB, 45 mM glycine, and 41mM NaCl adjusted to pH 3.0 with 1 M HCl.

Statistical analysis

The Kruskal-Wallis’ test was used for statistical analysis. Statistical differences between the groups were evaluated by the U Mann-Whitney’s test. The minimal level of significance was p<0.05.

RESULTS

In the first part of the study the effectiveness of hypothyroidism state was evaluated. Hence, the levels of fT3 and fT4 were markedly lower (p<0.01) in experimental hypothyroid rats (Tx and MTU) as compared with controls (Ctr and sham-Tx, Fig. 1). Moreover, TSH concentration in the plasma of rats with hypothyroidism (Tx and MTU) was strongly elevated (p<0.001) in relation to the two controls (Ctr and Sham-Tx; Fig. 2).

The measurements of collagen and GAG contents in the myocardium show that hypothyroidism independently on the method of its induction increases level of those compounds in the muscle of both right and left ventricles. Thus, in the left ventricle (Fig. 3) collagen level in thyroidecomised (Tx: p<0.05) and MTU treated rats (p<0.01) was increased. However, in the right ventricle (Fig. 4) marked elevation of collagen level was seen mainly in MTU treated animals (p<0.001). The GAG contents in the left ventricle (Fig. 5) were also elevated in
thyroidectomised (Tx: p<0.001) and MTU treated rats (p<0.02). Higher levels of GAG in the right ventricle (Fig. 6) were similarly observed in groups of animals with hypothyroidism induced by thyreoidectomy (p<0.001) and methylthiouracil (p<0.06) in comparison with controls.

Ultrastructural findings: The shape of the cultured cells observed under the electron microscope was typical for fibroblasts (Fig. 7). They were spindle-shaped, long and thin. Sometimes small cytoplasmic processes were visible at the end of cells. The nuclei were oval with morphological features of the high metabolic activity. They were half full with euchromatin and contained thin marginal zone of heterochromatin close to the nuclear envelope. Their nucleoli were spongiform type with well visible granular and fibrilar components, which is indicative of its very high transcriptional activity. The cytoplasmic organelles were in general typical for fibroblasts (Fig. 8). The granular endoplasmic reticulum (R) was well developed and showed marked dilatations. Its cisterne contained a medium dense, homogenous substance. The Golgi zone (Gc) was large and well developed with dilated dictiosomes and numerous vacuoles and vesicles. Mitochondria (M) were large but scarce. Close to the cellular membrane fascicle of thin fibers were seen. They made continuous layer on the one side and whole length of the cell. We also observed small vesicles (C), lying between cell membrane and cytoplasmic fiber bunches. They were closely connected with cell membrane and sometimes opened to the outer space. The presence of fibrilar structures and small vesicles connected with cell membrane suggests that fibroblasts started to differentiate into the myofibroblasts (Fig. 7 and 8).

In cell cultures, application of TSH in concentration ranging from 0.002 to 20 mIU/ml markedly increased the total cell number in the culture in parallel to the increased TSH concentration in the medium (Fig. 9). Statistically significant effects were seen at the TSH concentration of 2 mIU/ml (p<0.005) and 20 mIU/ml (p<0.05). Wound healing assay showed that TSH does not change the migration of fibroblasts isolated from rat’s heart in the culture (data not shown). TSH did not influence collagen and GAG levels in the culture of myofibroblasts isolated from the heart (data not shown).

**DISCUSSION**

Both thyreoidectomy and methlythiouracil treatment caused primary hypothyroidism. Thus, low level of fT3 and fT4 and elevated concentration of TSH were shown in rats with hypothyroidism (Fig. 1 and 2). The results discussed above...
clearly showed that applied experimental models of primary hypo- 
thyroidism are sufficient and comparable. Increased collagen content in the heart of hypothyroid rats was found in the two applied models (Fig. 3 and 4). These data are consistent with morphological observations indicating increased fibrosis in the heart interstitium of patients with hypo- 
thyroidism (28). However, our results do not explain the mechanism of that process. The increase of the collagen content in the heart is not an effect of direct stimulation of heart myofibroblasts by TSH. However, earlier studies provided some explanations of collagen metabolism changes by thyroids hormones. In hypothyroid patients increased level of telopeptides, markers of collagen synthesis (29), was reported. Administration of thyroid hormones to rabbits (30) or rats (31) induced hypertrophy of cardiac muscle but excess accumulation of collagen was not observed. Treatment of rats (32) with thyroxin resulted in accelerated collagen catabolism and elevated concentration of hydroyproline in serum and urine. It has been shown that, thyroid hormones accelerate parallely two opposite processes: collagen synthesis and catabolism but did not change collagen content in the heart. In hypothyroid subjects contradictory data were obtained. Short, thin and weak collagen fibers were found in wounds of patients with hypo- 
thyroidism (33). In hypothyroid rat ovary, procollagen I and III levels were increased (9). Contrary to that, hypo- 
thyrosisis prevents the liver fibrosis in rats. This effect is caused by triiodothyronine deficiency the hormone stimulating collagen synthesis and reducing metaloproteinase-2 secretion (34). Contradictory effects of hypo- 
thyroidism on collagen accumulation were dependent on target organ and experimental model. Augmentation of collagen content in the heart of hypothyroid rats was presumably dependent on reduction of this protein catabolism (35).

The present results clearly show the increased content of GAG in the heart of rats with experimental primary hypo- 
thyroidism (Fig. 5 and 6). This effect did not depend on the method of hypo- 
thyroidism induction. The influence of hypo- 
thyroidism on GAG metabolism remains the matter of debate. Suppression of proteoglycan degradation in epiphyseal cartilage was found in rats with hypo- 
thyroidism. This phenomenon was reversed by thyroxine injections (36). Moreover, thyroid hormones stimulate aggrecan breakdown in growth plate cartilage of rats (37). The hyaluronic acid content elevation was seen in the hindlimb skin and skeletal muscle of hypo- 
thyroid rats. These changes influenced on interstitial fluid balance (38). Thyroid hormone deficiency increased level of GAG in cultures of human skin fibroblasts (39) and in the human heart according to morphological studies (28). Bovine TSH (Sigma) was used in the present study. Bovine TSH is an independent mitotic factor for rat cells (19). The mitogenic effect of bovine TSH was proved for thyroid epithelial cells of rat. Both pure rat and bovine TSH increased rat thyroid epithelial cell number in culture. However, bovine TSH expressed larger effect. Incorporation of [3H] thymidine into cellular nuclei of rats was augmented by bovine TSH (19).

The observation of the spindle shape, typical cytoplasmic organelles and fibrilar structures of the isolated cells from hearts allows identifying cells as myofibroblasts (40). Bovine TSH increased myofibroblasts number in the cultures. As the neocritic cell number was not modified by TSH, the results suggest that elevated cell number is rather the effect of increased proliferation. Isolated heart myofibroblasts are the main source of extracellular compounds such as GAG and collagen. Earlier data showed that thyrotropic hormone injected into mice or rats induced proliferation of the connective tissue cells and increased deposition of both collagen and proteoglycans in the retrobulbar space (41). Several studies documented the existence of TSH receptors in the human fibroblasts of skin, retrobulbar space and pretilial area (21, 22). TSH receptor transcripts (23) and tyroptropin receptor immunoreactivity (42) were found in the human pretilial connective tissue and fibroblasts cultured in vitro. Our study showed that TSH did not change both collagen and GAG content in cultures of myofibroblasts isolated from the heart. Thus, increased extracellular matrix content in the heart of hypo- 
thyroid rats is not dependent on a direct TSH action on heart myofibroblasts.

CONCLUSION

Our study showed increased accumulation of collagen and glycosaminoglycans in the heart of rats with experimental primary hypo- 
thyroidism. In cultures of myofibroblasts isolated from rats heart, TSH increased cells number but had no influence on GAG and collagen contents. Augmentation of cells number is the result of direct stimulation of myofibroblasts by TSH. The increased level of collagen and GAG in hearts of rats with primary hypo- 
thyroidism is not dependent on direct effects of TSH on myofibroblast of heart.

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