EKSPERIMENTINIAI TYRIMAI

Application of Photoshop-based image analysis and TUNEL for the distribution and quantification of dexamethasone-induced apoptotic cells in rat thymus

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Key words: *dexamethasone, thymus, apoptosis, TUNEL, morphometry.*

Summary. *The aim of the present study was to determine the target site cells in the rat thymus after exposure to the synthetic glucocorticoid, dexamethasone, at therapeutic doses. The findings of histology and histochemistry (Feulgen, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling – TUNEL) with quantification by computerized histomorphometry are described.*

Material and methods. A quantified investigation of apoptotic and mitotic thymic lymphocytes in 36 young adult Wistar rats was performed at 1–7 days after a 3-day injection of dexamethasone (a total dose of 1.2 mg/rat intraperitoneally).

Results. At the first day after dexamethasone administration the moderate involution and atrophy of thymus histology were observed with simultaneous fall in cortical cellularity and mitotic activity of thymocytes. More rapid fall appeared in the inner cortex. The number of apoptotic (TUNEL-positive) cells was significantly increased. On the days 5 and 7 the expression of apoptosis and the cell proliferation were at almost normal level.

Conclusions. The findings suggest that dexamethasone-induced apoptosis of cortical thymic lymphocytes, mainly correlated with synchronous inhibition of mitosis and cell number fall in thymus. The main target sites of dexamethasone injury were cells in the inner cortex of lobuli thymi.

Introduction

Apoptosis (programmed cell death or "cell suicide") is a general biological mechanism responsible for the maintenance of permanent cell number. The impairment of apoptosis regulation entails disorders of homeostasis and various diseases. This explains biological and medical importance of apoptosis (1–4).

It is generally accepted that the thymus is vulnerable to adverse effects of immunotoxic chemicals (5–7). Thymic lymphocytes are sensitive to chemicals such as glucocorticoids and tend to undergo apoptosis with thymic atrophy (8, 9).

Apoptosis is characterized by cell shrinkage, condensed cytoplasm, lucent areas around chromatin, chromatin condensation at the periphery of non-fragmented nuclei, appearance of apoptotic bodies and DNA fragmentation (10–14). Thymocyte apoptosis plays a physiological role in the T-cell selection in the thymus (15).

General methods for apoptosis study are routine histology and histochemistry by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). The induction of thymocyte apoptosis by dexamethasone is one of the best-characterized experimental models of programmed cell death (16). However, the morphology, distribution, and quantification of the thymocyte apoptosis evoked *in vivo* by dexamethasone are largely unknown at present (14). Apoptotic changes in cellular structure such as condensation of the nuclear (chromatin) and cytoplasmic structures (especially mitochondria), blebbing of the cell membrane, characteristic swelling of the endoplasmic reticulum, and fragmentation of the cells in membrane bound apoptotic bodies, are the dramatic signs of total cell destruction (17).

The aim of present study was to determine the target site in the rat thymus after exposure to the synthetic glucocorticoid, dexamethasone (DEXA), at therapeutic doses. This paper presents the findings of histological examination with morphometry, histoche-

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mistry with Feulgen for cell proliferation and TUNEL for the detection and quantification of apoptotic cells.

Material and methods

In the research 36 young adult male Wistar rats (200–220 g) were used. The animals were investigated on days 1 to 7. The guidelines for the care and use of the animals were approved by the Ethical Committee of the University of Tartu.

The animals (housing, treatment, protocol)

The animals were housed in an animal room (in special boxes for three animals in each) controlled at temperature of 22±2°C and humidity of 55±15% with more than 10 air changes per hour (all-fresh-air system) and light/dark cycle of 12 h (lights on: 07:00–19:00). They were fed a certified diet ("Dimela" – Finland R-70 and R-34) and water ad libitum. Animals were acclimated for seven days before dosing.

The rats were injected with 1.2 mg of DEXA per rat intraperitoneally on days 1, 4, and 7 and sacrificed 1, 2, 3, 5 and 7 days after last injection (n=6 in each group). The animals were decapitated under deep anesthesia with intramuscular injection of 50 mg/kg ketamine and 5 mg/kg diazepam.

The thymus was carefully removed and dissected free of accompanying tissue. Approximately one-half of each thymus was snap-frozen in liquid nitrogen and stored at –80°C until TUNEL analysis. The remaining part of the thymus was used for routine histology.

Histology

The thymus was fixed in 10% buffered formalin and embedded in paraffin; thick sections of 7 μm were stained with hematoxylin–eosin and Heidenhain's iron hematoxylin for the detecting of dark (mainly apoptotic) lymphocytes by histomorphometry. The sections for mitotic cell study were stained with Feulgen. The cells in 10 microscopic fields were counted per animal, and mitotic index (MI) was assessed (number of mitoses per 1,000 non-divided cells).

Histomorphometry

Morphometric assessment was conducted to detect objectively the apoptotic changes in the cortical and medullary cells in the thymus using a new method of computerized morphometry described previously (18). The pictures of slices were photographed by a light-microscope Olympus BX-50. The further process was performed with a computer program, Adobe Photoshop 5.0, under a simultaneous visual control of light microscopy. The pictures were analyzed with Adobe Photoshop; the areas of thymus were selected, and thymic lymphocytes were observed in different colors; percentage of dark cells (mainly apoptotic) was measured histomorphometrically. The painted areas were summarized in pixels, and the proportions of different cells were expressed as percent of total cell number. Computerized histomorphometry is widely used in the quantitative analysis of tissues and cells (19–22).

TUNEL histochemistry

Detection of apoptotic cells by TUNEL. Frozen tissue sections were stained for TUNEL *in situ* using a standard cell death detection kit POD (Roche, U.S.A.), according to the manufacture guidelines (6, 23–27). Each experiment set up by TUNEL reaction without terminal transferase served as a negative control.

To define the distribution of apoptosis within the thymus cortex, a direct cell count was performed on TUNEL sections using a microscope to count the number of TUNEL-positive cells in 10 microscope fields (12,800 μm²) per animal at a magnification of 400× (6). Results are expressed as apoptotic cell area / total cell area in percentage (pixels).

Statistics

Statistical analysis was performed using one sample t-test and the unpaired t-test (GraphPad Quick Calcs: analyze continuous data) at the 0.05 level of significance ($p<0.05$) with the Newman-Keuls multiple comparison.

Results

The apoptotic cells (APCs) of the thymus have a certain distribution within lobes, detected by new Photoshop analysis method. Dark cells (mainly apoptotic) concentrated largely in the inner cortex of thymus lobes (Fig. 1-1, 1-2).

At the first day after DEXA injection the moderate atrophy with accidental involution of thymus was observed, and there was a simultaneous fall in cortical cellularity and mitotic activity of thymic lymphocytes (Table 1 and 2, Fig. 2-1, 2-2).

More rapid fall occurred in the inner cortex (Fig. 3-1, 3-2, 3-3, 3-4). A statistically significant decrease in cell number from 383±27 to 197±25 and in mitotic index (MI) from 3.2±0.3 to 1.3±0.4 was observed.

The number of APCs and TUNEL-positive cells was the highest (Table 3 and 4, Fig. 4-1, 4-2). An increase in the number of apoptotic cells from 6.3±0.8 to 48.0±1.9 and in the number of TUNEL-positive cells from 24.6±2.2 to 86.3±7.2 was seen. Medullar layer of lobuli was not changed.

On day 2 in the outer cortex and day 5 in the inner

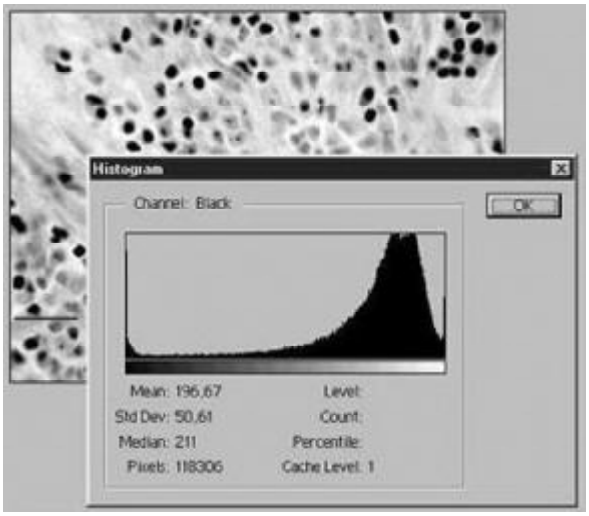

Fig. 1-1. **The part of the thymus cortex in rat**
Dark mainly apoptotic cells and light thymic lymphocytes. Histogram contains data on the whole field (in pixels 118,306). Hematoxylin and eosin. Bar: 50 μm.

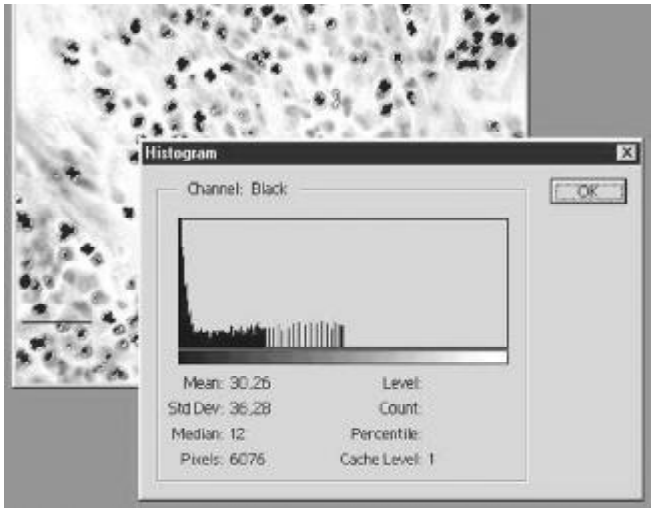

Fig. 1-2. **The part of the thymus cortex in rat**
Dark cells with selected nuclei. "Inversion" of histogram. The number of selected apoptotic cell nuclei on the whole field is 6,076 pixels. Hematoxylin and eosin. Bar: 50 μm.

Table 1. **The number of lymphocytes in rat thymus 1–7 days after the dexamethasone (DEXA) injection at a dose of 1.2 mg/rat intraperitoneally (per standard ocular lens network 8×8 mm±SEM)**

Days after the last DEXA injection	Outer cortex	Inner cortex	Medulla
1	252±25*	197±25*	212±34
2	346±53	201±16*	231±46
3	385±28	237±38*	227±44
5	375±38	278±24	236±39
7	396±40	265±32	239±22
Control	446±64	383±27	243±42

* $p<0.05$ as compared to control.

Table 2. **The mitotic activity of lymphocytes in rat thymus 1–7 days after the dexametazone (DEXA) injection at a dose of 1.2 mg/rat intraperitoneally (number of mitoses per 1,000 cells±SEM)**

Days after the last DEXA injection	Outer cortex	Inner cortex	Medulla
1	1.4±0.2*	1.3±0.4*	1.7±0.2
2	2.6±0.4	1.2±0.2*	1.8±0.2
3	2.3±0.3	1.8±0.2*	2.1±0.2
5	2.9±0.3	2.2±0.6	2.2±0.2
7	3.1±0.3	2.8±0.5	2.0±0.2
Control	3.3±0.2	3.2±0.3	2.1±0.3

* $p<0.05$ as compared to control.

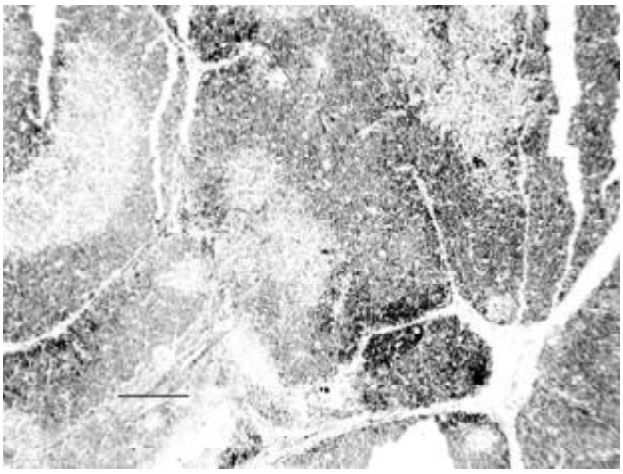

Fig. 2-1. **Thymus in rat**
Thymus lobe with peripheral cortex (dark) and central medulla (light stained). Hematoxylin and eosin. Bar: 500 μm.

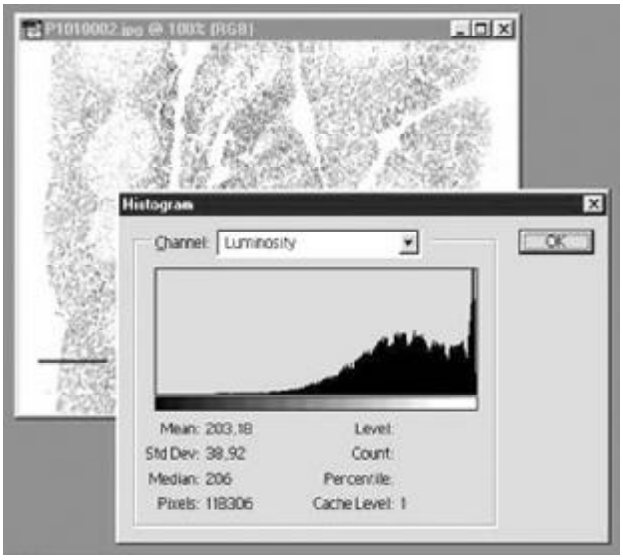

Fig. 2-2. **Thymus one day after dexamethasone injection intraperitoneally in rat**
The moderate atrophy of cortex tissue with involution of lobe. Histogram with whole field pixels (118,306). Hematoxylin and eosin. Bar: 500 μm.

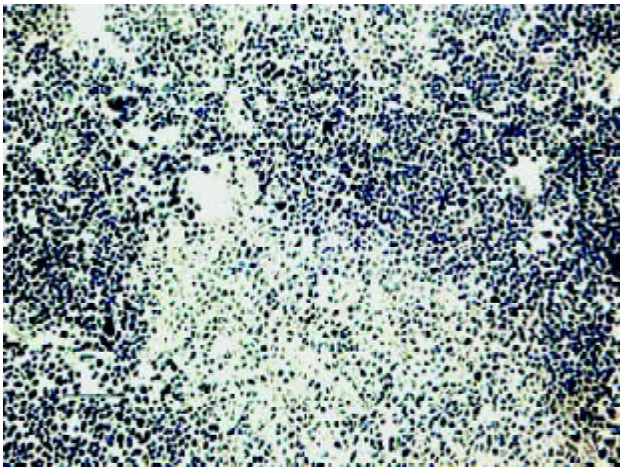

Fig. 3-1. **The part of the thymus lobe in rat with dark stained cortex and light stained medulla**
Iron hematoxylin. Bar: 100 μm.

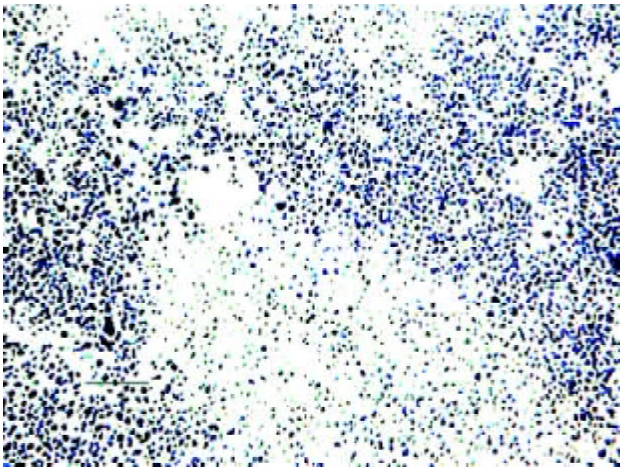

Fig. 3-2. **The part of the thymus lobe in rat**
Color selected apoptotic cells, mainly in the inner cortex. Iron hematoxylin. Bar: 100 μm.

cortex the number of cortical thymic lymphocytes and their mitoses did not differ from control values, whereas both the outer and inner cortex had a significantly higher number of apoptotic and TUNEL-positive cells till day 5 (Table 3 and 4).

Apoptotic cells were mainly distributed diffusely. On the first day of DEXA injection some apoptotic cells in the inner cortex were detected as aggregates ("clusters") (Fig. 5-1, 5-2). On the fifth day, the appearance of cell death became infrequent, and a marked increase of cellularity and mitotic activity was observed. On day 7, the expression of apoptosis and the cell proliferation were almost at normal level. The findings suggest that DEXA-induced apoptosis which mainly correlated with simultaneous inhibition of mitosis and certainly with the cell number in thymus. The inner cortex is the most sensitive part of thymus to DEXA injury.

Discussion

Apoptosis is the physiological way for nucleated cells to die. Autoreactive T and B cells, millions of which are produced by the immune system every day, are also eliminated by apoptosis, ensuring the steady

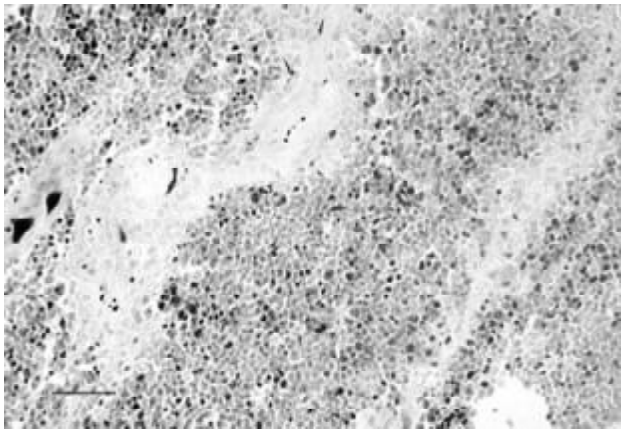

Fig. 3-3. **The part of the thymus cortex one day after dexamethasone injection intraperitoneally in rat**
The moderate atrophy of cortex tissue with involution of lobe. Iron hematoxylin. Bar: 100 µm.

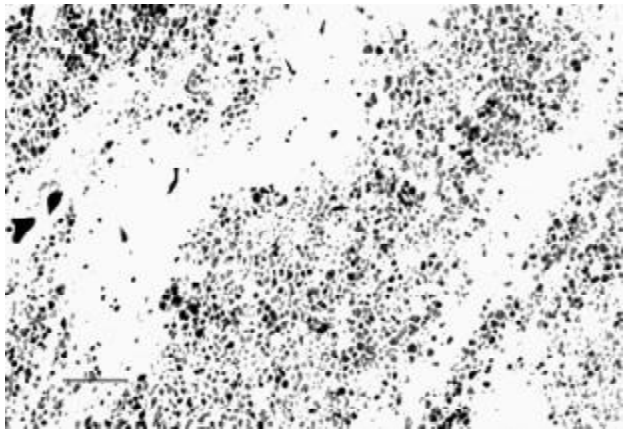

Fig. 3-4. **The part of the thymus cortex one day after dexamethasone injection intraperitoneally in rat**
The fall of the cellularity in the inner cortex of lobe. Diffuse and "cluster" distribution of apoptotic cells. Iron hematoxylin. Bar: 100 µm.

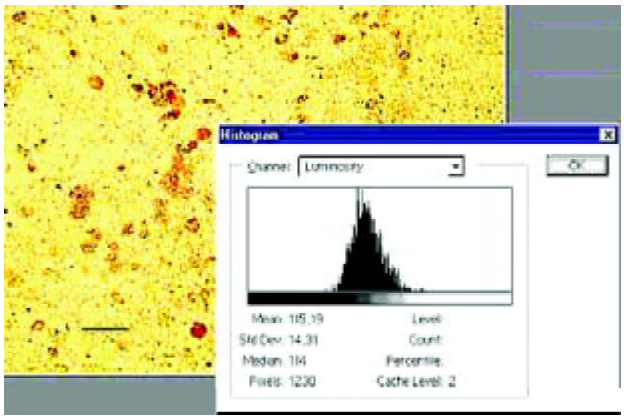

Fig. 4-1. **The part of the thymus cortex in rat**
Histogram for the TUNEL-positive cells (1,230 pixels). Iron hematoxylin. Bar: 100 µm.

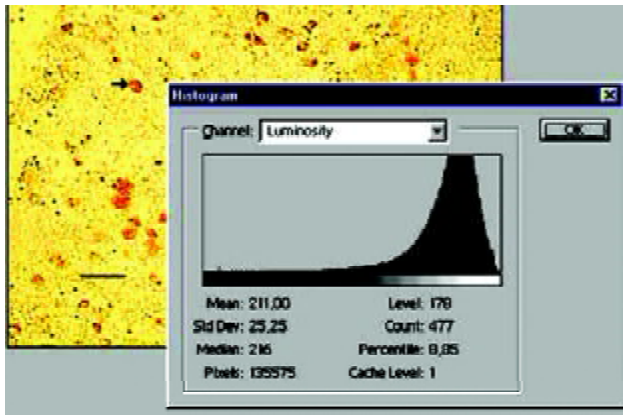

Fig. 4-2. **The part of the thymus cortex one day after dexamethasone injection intraperitoneally in rat**
The large number of the TUNEL-positive cells. Diffuse and "cluster" distribution of apoptotic cells *in situ*. Histogram on the whole field (135,575 pixels). Arrow – TUNEL-positive cell. Iron hematoxylin. Bar: 100 µm.

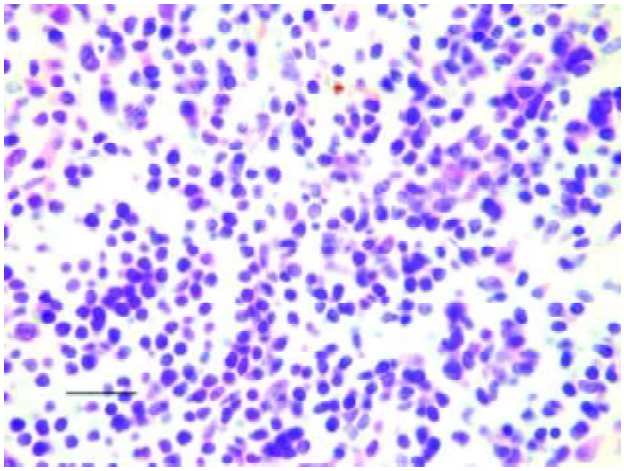

Fig. 5-1. **The inner cortex of the thymus lobe one day after dexamethasone injection intraperitoneally in rat**
Iron hematoxylin. Bar: 50 µm.

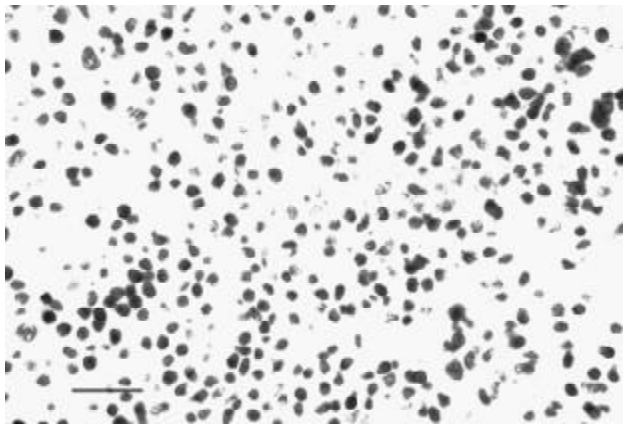

Fig. 5-2. **The inner cortex of the thymus lobe one day after dexamethasone injection intraperitoneally in rat**
Color selected diffuse and aggregates ("clusters") of apoptotic cells. Iron hematoxylin. Bar: 50 µm.

Table 3. **The percentage of apoptotic cells in rat thymus 1–7 days after the dexametazone (DEXA) injection at a dose of 1.2 mg/rat intraperitoneally (percentage of total cell area±SEM). Computer field in use: 16.7%; 118,306 pixels (=100%)**

Days after the last DEXA injection	Outer cortex	Inner cortex	Medulla
1	36.2±2.6*	48.0±1.9*	2.3±0.3
2	14.5±0.5*	37.6±1.8*	1.7±0.2
3	15.3±1.2*	28.1±1.2*	1.9±0.2
5	8.3±0.6	13.4±0.5	2.4±0.3
7	5.3±0.6	9.2±0.7	1.6±0.1
Control	3.6±0.5	6.3±0.8	1.2±0.1

* $p<0.05$ as compared to control.

Table 4. **The number of TUNEL-positive cells in the cortex and medulla of rat thymus 1–7 days after dexametazone (DEXA) injection at a dose of 1.2 mg/rat intraperitoneally (per standard ocular lens network 8×8 mm±SEM)**

Days after the last DEXA injection	Outer cortex	Inner cortex	Medulla
1	75.2±6.4*	86.3±7.2*	5.5±0.9
2	52.3±5.0*	83.7±6.0*	4.1±1.2
3	51.6±4.3*	74.4±5.2*	5.3±1.1
5	31.4±3.2	34.2±7.1	5.7±1.0
7	19.8±5.2	26.5±6.4	4.2±1.2
Control	21.2±3.7	24.6±2.2	3.1±0.6

* $p<0.05$ as compared to control.

state or homeostasis in the organism. Recently dysregulation ("too much or too little") of apoptosis has emerged as a new concept to explain important features in the development of several as yet poorly understood diseases. Unregulated excessive apoptosis may be a cause of various degenerative and autoimmune diseases (multiple sclerosis, type 1 diabetes mellitus, Hashimoto thyroiditis, Sjögren syndrome, and certain cancers such as melanoma) that are characterized by an excessive loss of normal or protective cells (24). Endogenous glucocorticoids (GCs) take part in fast type homeostasis. The administration of exogenous GCs depressed the immune system and caused thymic atrophy, inhibition of mitotic activity in cortical thymic lymphocytes and decrease in the number of cells.

Synthetic glucocorticoid, dexamethasone, administered at a therapeutic dose of 1.2 mg/rat intraperitoneally on days 1, 4, and 7 before the decapitation of the animals, caused thymus atrophy of "delayed" type (28), correlated with the inhibition of cell mitotic division, and enhanced the number of apoptotic cells, especially in the inner cortex.

In our experiments percentage of APCs in the outer cortex was 36.25%, in the inner cortex – 48.0% (these cells are more sensitive to GCs). TUNEL-positive cells comprised 29.3% and 43.5%, respectively (counted per standard ocular lens of light microscope). The number of APC was higher than the number of TUNEL-positive cells, because the group of apoptotic cells consisted of dark apoptotic cells, some necrotic and mitotic cells (5, 29, 30) which were not differentiated by Photoshop-based image analysis. The necrotic cells and mitoses were differentiated and selected by immersion. The TUNEL-positive cells are apoptotic cells *in situ*, because the chromosomal nick ends are direct labeled (6, 14, 16, 27). Thus, indirect detection of APCs by computerized histomorphometry, correlated with TUNEL assessment, is quite correct for apoptotic thymic lymphocyte measurement. On the

contrary, measurement of optical density (OD) in flow cytometry, without direct evaluation of apoptotic cell quality, is useless for morphology (27). The specificity of TUNEL-positive cells varied with tissue and degree of injury. After therapeutic doses of DEXA TUNEL-sensitivity of thymic lymphocytes is 100% in correlation with histology. TUNEL specificity after predominantly necrotic injury did not exceed 70% of detected apoptotic cells (31). In mouse kidney and liver (but not in lymphoid organ, spleen), when examining apoptosis by TUNEL assay, the apoptotic signal may be false-positive (32). The cause of the false-positive signal remains unknown.

The number of mitoses and APCs, especially when APCs are expressed as a percentage of the total number of lymphoid cells, is a marker of thymus proliferative rate and widely used in biofunctional staging, based on cell kinetics in thymus (33–36). It is a clinicopathological staging of GCs effect on thymus.

The results of different studies of thymic lymphocyte apoptosis are heterogeneous and vary widely. The percentage of spontaneous APCs in well-nourished rats was 1.9±1.0, and the frequency of DEXA-induced apoptosis was 47.9±10.1% (16). Our study showed that the percentages of apoptotic cells in the outer cortex, the inner cortex, and medulla of lobuli thymus were 36.2±2.6, 48.0±1.9, and 2.3±0.3, respectively. The sensitivity of thymic lymphocytes to DEXA-induced apoptosis varies with exo- and endogenous factors (37, 38). Apoptosis machinery is too much complicated. DEXA-induced apoptosis in thymus requires a sequence of events including interactions with the GC receptor, phosphatidylinositol-specific phospholipase C, activation of acidic sphingomyelinase (39–41). At last, general apoptosis-induced event is a genetically determined degradation of cellular mRNA. This degradation also occurs *in vivo*, specifically during thymocyte apoptosis (early apoptotic event that may become a new hallmark of apoptosis).

Conclusions

The synthetic glucocorticoid, dexamethasone, administered at therapeutic doses (a total dose of 1.2 mg/rat injected per three days intraperitoneally) caused the moderate involution and atrophy of thymus, which was correlated with synchronous inhibition of mitosis and decrease in cell number. More rapid fall appeared in the inner cortex.

The number of dark apoptotic cells 1–3 days after dexamethasone administration in iron hematoxylin stained slides as well as the number of TUNEL-positive cells in special slides was significantly increased. The main target site of dexamethasone injury was the inner cortex of lobuli thymi.

The effect of dexamethasone on the thymus is reversible. Five days after cessation of drug administration the thymus histology and histochemistry were restored up to normal level.

The results of indirect detection of apoptotic cells by computerized histomorphometry in iron hematoxylin slides correlated with the results of direct measurement of apoptotic cells by TUNEL assay.

Deksametazono sukeltų apoptozinių ląstelių pasiskirstymo ir ląstelių skaičiaus žiurkių užkrūčio liaukoje nustatymas remiantis nuotraukų analize „Photoshop“ programine įranga bei TUNEL metodu

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Raktažodžiai: deksametazonas, užkrūčio liauka, apoptozė, TUNEL metodas, morfometrija.

Santrauka. *Tyrimo tikslas.* Nustatyti žiurkių užkrūčio liaukos pažaidos vietas, injekavus sintetinio gliukokortikoido, deksametazono, gydomosiomis dozėmis. Pateikiami histologiniai ir histocheminiai (Feulgen, TUNEL – angl. *terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling*) bei ląstelių skaičiaus įvertinimo kompiuterine histomorfometrija duomenys.

Tyrimo medžiaga ir metodai. Kiekybiniai apoptozinių ir mitozinių užkrūčio liaukos limfocitų tyrimai atlikti su 36 Wistar veislės jaunais suaugusių žiurkių patinais praėjus 1–7 paroms po trijų deksametazono injekcijų į pilvo ertmę (bendra dozė – 1,2 mg žiurkei).

Rezultatai. Praėjus parai po deksametazono suleidimo, pastebėta nežymi užkrūčio liaukos involiucija bei sunykimas ir ląstelių skaičiaus bei timocitų mitozinio aktyvumo sumažėjimas. Užkrūčio vidiniuose liaukos žievės sluoksniuose vyksta greitesni pokyčiai. Apoptozinių (TUNEL teigiamų) ląstelių skaičius reikšmingai

padidėjo. 5–7 parą vyksta normali apoptozė ir ląstelių dalijimasis.

Išvados. Tyrimo duomenimis, deksametazonas sukelia užkrūčio liaukos žievės limfocitų apoptozę ir kartu slopina mitozę bei mažina ląstelių skaičių užkrūčio liaukoje. Pagrindinė deksametazono sukeltos pažaidos vieta – vidiniai užkrūčio liaukos žievės sluoksniai.

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