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Effect of the inflammatory environment and pravastatin on skeletal muscle regeneration

by Karolina Cseri

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Introduction

Myocardial infarction and ischemic heart disease were almost completely unknown before the 1900s. More and more cases were reported after 1920, and by the 1930s it was already causing a serious public health problem. According to statistics, there is currently one heart attack in America every tenth of a second. 50,000 people die of heart attacks in Hungary every year. Today, it is a widely accepted view that heart loss is due to a sedentary lifestyle, excessive food intake, over-consumption of refined foods, and poor diet. There are opponents of the cholesterol-raising effect of diet and the theory of the link between high blood fat and heart mortality, but the popularity of cholesterol-lowering statins has been unbroken for years. Their risk lies in the fact that statins, in addition to blocking the own cholesterol synthesis of the cells, also affect metabolism in other ways, self-regulatory mechanisms that go beyond our current knowledge. As a side effect, autoimmune muscle involvement or fatal renal failure is becoming more common.

Idiopathic inflammatory myopathies

Idiopathic inflammatory myopathies (IIMs) include heterogeneous groups of neuromuscular diseases commonly referred to as myositis. For decades, these were divided into three subgroups based on the presence and severity of muscle weakness, myositis, and extramuscular symptoms. In 1975, following the work of Bohan and Peter, only two groups were considered, polymyositis (PM) and dermatomyositis (DM), and in 1995, Griggs et al. Also described inclusion body myositis (IBM) as a third group. In 2003, an international conference (European Neuromuscular Center International Workshop - 2003 ENMC-IIM) expanded the existing categories with immune-mediated necrotizing myopathy (IMNM) and non-specific myositis. Based on the research results of the past decade, the discovery of myositis-specific antibodies (MSA), and extensive clinical experience, requires to develop a new classification system. Formulating a diagnosis, the EULAR / ACR (European League Against Rheumatism / American College of Rheumatology) classification criteria should be considered first, and then the subgroup can be defined based on a multi-step system of criteria. The current categorization distinguishes six subgroups: polymyositis (PM) - which has become a subset of immune-mediated necrotizing myopathy (IMNM), inclusion body myositis (IBM), amyopathic dermatomyositis (ADM), dermatomyositis (DM), and juvenile dermatomyositis (JDM). If the symptoms meet the criteria set for EULAR / ACR IIM, the next consideration is the age at the onset of the first symptom. If complaints occurred under the age of 18, juvenile dermatomyositis can be diagnosed in the presence of skin symptoms, and juvenile myositis in the absence of skin symptoms. If there is no skin symptom, but the patient complains of weakness of the flexor muscles of the fingers that does not respond to therapy, or rimmed vacuoles are confirmed on the muscle biopsy samples, then the diagnosis is inclusion body myositis (IBM).

If the stubborn weakness of the flexor muscle is not characteristic, and the presence of rimmed vacuole is not confirmed histology, the diagnosis is polymyositis. Over the age of 18, when skin symptoms also occur, the question is whether there is a symmetrical and progressive weakness in the upper or lower limbs, or a weakening of the neck flexor muscles relative to the neck bending muscle, or a proximal weakness of the lower extremities relative to the distal regions. If at least one of these symptoms is present, the diagnosis is dermatomyositis; in the absence, the diagnosis is amyopathic dermatomyositis.

Cholesterol

Cholesterol is a lipid-based, sterane framed organic molecule. It is an essential component of the membrane of human and animal cells. Researchers were initially skeptical about the link between cholesterol and coronary heart disease, but later more and more studies have been published that have demonstrated the role of cholesterol in the development of cardiovascular disease. Cholesterol serves as a precursor for the biosynthesis of bile acids, steroid hormones, and vitamin D. Every cell in the body is able to synthesize, and in vertebrates it is produced in the highest amount by liver cells. Subsequent studies have shown that mortality from coronary heart disease is mainly attributable to low-density lipoprotein (LDL) cholesterol, which accounts for 70% of total cholesterol. a theory has emerged that elevated total, more specifically LDL, cholesterol levels can cause coronary artery disease. At present, millions of people in developed countries and in Hungary are killed by cardiovascular diseases caused by atherosclerosis, which affect society not only with premature death but also with long years of disability.

Statins

Statins are the most effective, widely used drugs in hypercholesterolemia for reducing the level of low-density lipoprotein cholesterol and triglycerides by inhibiting 3-hydroxy-3methyl coenzyme a (HMG-CoA) reductase thus blocking the key enzyme of the synthesis of cholesterol. Beside their therapeutic advantages, statins may rarely induce serious muscle related adverse effects. Statininduced myopathy, according to its pathogenesis, can either be toxic non-autoimmune or statin-induced necrotizing autoimmune myopathy (sinam). Toxic myopathy can be focal or generalized, severity of symptoms is dose-dependent. SINAM is a rare and severe form of druginduced myopathy in which statin treatment initiates an iutoimmune process which cannot be reversed by drug withdrawal. We have previously shown that statin treatment decreased the proliferation and fusion of skeletal myotubes in cell culture and negatively regulated calcium homeostasis of adult skeletal muscle fibers. These effects can contribute to the observed myopathies in statintreated patients. Besides the direct action of statins on muscle cells, their effects on the macrophages may also play a pathogenic role in statin-induced myopathy.

High mobility group box 1 protein (HMGB1)

In case of senior patients, the appearance of muscle symptoms is initiated by statin therapy to lower cholesterol. In some cases, discontinuation of treatment does not resolve the complaints, as the symptoms are perpetuated by autoimmune processes. Molecules released from damaged cells, such as HMGB1, continue to trigger inflammation. Thus, HMGB1 may play a pathogenic role in the development of myositis, muscle weakness, and muscle pain in autoimmune diseases such as idiopathic inflammatory myopathies or rheumatoid arthritis. “High Mobility Group” proteins are non-histone type chromatin proteins that are present in all cell types. It is actively released from living cells and passively from dead cells. In the extracellular space, it can activate the natural (innate) immune system by providing a warning function. Large amount of HMGB1 released into the extracellular space has an inflammatory effect. HMGB1 consists of two positively charged DNA binding subunits (boxes A and B) and a negatively charged acidic terminus. It contains two nuclear localization signal segments (amino acids NLS1: 28-44; amino acids NLS2: 179-185) that play an important role in the transport of the protein to the nucleus. The 50-183 amino acid sequence is responsible for RAGE binding. Extracellular cytokine activity is located in box B and can be antagonized by the cleaved box A subunit. The cysteine at position 106 in box B is essential for HMGB1 to activate cytokine release. The transcriptional stimulating effect of the protein is provided by the C-terminal acidic terminus. Currently known, proven receptors are toll-like receptor-4 (TLR-4) and RAGE (receptor for advanced glycation end products). Many receptor systems have been associated with HMGB1, but these are presumably not specific for HMGB1 but for molecules complexed with HMGB1. The effect of the relationship between HMGB1 and RAGE is currently the subject of intensive research. Overall, studies indicate that binding of HMGB1 to RAGE directly activates the NF-κB pathway and the resulting cytokine production. Both TLR-4 and RAGE receptors are found on macrophages, but if TLR-4 is functionally inactive or absent, none of the isoforms of HMGB1 can stimulate the cell to produce cytokines. Thus, it can be hypothesized that the HMGB1-RAGE complex is not able to directly induce cytokine production.

Aims and perspectives

-How does the in vivo inflammatory environment affect the ability of muscle satellite cells to proliferate and differentiate?

-What effect can in vivo methylprednisolone treatment have on myogenesis?

-How does corticosteroid treatment affect the ability of primary cultures initiated from myositis to divide and fuse?

It is a well-known phenomenon in the clinic that statin therapy causes chronic myopathy in some patients by initiating autoimmune processes. This experience raised several questions:

-How do macrophages and myogenic cells affect each other's proliferation in statin-induced myopathic muscle?

-Is pravastatin, as a known anti-inflammatory substance, able to reduce myositis?

- How does pravastatin affect the ability of myogenic cells to proliferate and differentiate?

Materials and methods

Experiments and examinations performed on striated muscle tissue of human origin

Ethical approval

The study protocol was approved by the Human Ethics Committee of Medical and Health Science Center, University of Debrecen. Written informed consent was obtained from the patients before they entered the study.

Biopsy specimens and muscle samples dissected in orthopaedic surgery

Biopsy specimens were obtained either from human subjects suffering from PM (n = 15) or DM (n = 6) and from osteoarthritic patients undergoing orthopaedic surgery (n = 12). Thirteen IIM patients of the 21 were not treated with corticosteroids before biopsy while eight patients received methylprednisolon (12–16 mg/day). The age of IIM patients varied between 23 and 74 years (52.7 ± 13.3; mean ± SD, n = 21), 3 men and 18 women participated in the study. The biopsy was carried out by surgical intervention under local anaesthesia from the altered proximal muscles (deltoid or quadriceps femoris, whichever was more affected in the given patient). Muscle samples from the gluteus medius were excised during surgical intervention directed to hip replacement in the patients with coxarthrosis (age: 65.3 ± 8.8, n = 12; male: 4, female: 8).

Primary cell culture of human skeletal muscle satellite cells

For mechanic and enzymatic dissociation of satellite cells from biopsy pecimens and for culturing. Briefly, the biopsy specimens were digested in a $Ca2^{+}$ and $Mg2^{+}$ free phosphate buffer with 0.75 mg/ml type 2 collagenase and trypsin. Dissociated cells were plated on glass coverslips in HAM's F12 growth medium containing 5 %foetal bovine serum (FBS) and 5 % horse serum (HS), and were kept at 37̊C in a 5 % CO2 containing atmosphere. After 4 days the cells were placed in a differentiating medium supplemented with 2 % HS and 2 % FBS for further 7 days to initiate differentiation.

Examination of cell proliferation and differentiation

Primary cultures of human striated muscle cells were imaged daily from the 2nd to the 11th day after plating using a Canon EOS-300D digital single lens reflex camera mounted on a phase contrast microscope. Five regions of interest per dish were imaged every day. Myogenic nuclei were marked manually and morphometric analysis was done on these images. Cell proliferation was characterised by the increase in the number of myogenic nuclei normalised to

the value at the 2nd day of culturing. Differentiation was assessed by fusion index (number of myogenic nuclei in the myotubes divided by the total number of the myogenic nuclei).

Immunohistochemistry for HMGB1

For immunohistochemistry (IHC) formalin-fixed, paraffinembedded muscle samples were used as described earlier (Kistama´s et al. 2013). Briefly, the HMGB1-specific primary antibody (diluted in phosphate-buffered saline containing 1 % bovine serum albumin, monoclonal anti-HMGB1 antibody raised in mouse, was used in a 1:500 dilution (final concentration 2 μg/ml). Following overnight incubation with primary antibody, sections were washed (phosphate-buffered saline, 3 9 10 min) and incubated with horseradish peroxidaseconjugated secondary antibody (15 min, room temperature, Super SensitiveTM One-Step Polymer-HRP Detection System). The reaction was visualized by VECTOR DAB peroxidase substrate kit according to the manufacturer's protocol. Haematoxylin staining was used for nuclei counter stain (10 s). Tonsil sections were used as a positive control. Negative controls were made using 1 % BSA without primary antibody.

Immunohistochemistry for CD45

Formalin-fixed, paraffin-embedded sections of human skeletal muscle were used for IHC. Paraffin sections were deparaffinized, rehydrated, and incubated in 0.3 % H_2O_2 in distilled water for 15 min. For antigen retrieving, the slides were incubated with Tris–EDTA buffer (10 mM Tris Base, 1 mM EDTA solution, 0.05 % Tween 20, pH 9.0) in boiling pressure cooker (100℃) for 20 min. To reduce non-specific binding, slides were incubated in 5 % BSAPBS. Tissue sections were incubated with monoclonal mouse anti-human CD45, in 2.5 % BSA-PBS at a 1:50 dilution for 1 h at room temperature in a humidity chamber. 2.5 % BSA-PBS served as a negative control for secondary antibody. Peroxidase-labelled polymer conjugated to goat anti-rabbit immunoglobulin antibody was used as secondary reagent. The microscopic visualization was made using the VECTOR VIP peroxidase substrate kit.

Western blot analysis

Total protein content of tissue lysates were examined by western blot analysis. Dissected muscle pieces were frozen in liquid nitrogen immediately and homogenised in a mortar with lysis buffer (20 mM Tris–HCl, 5 mM EGTA, and pH 7.5). Tissue lysates were ultrasonicated (40–50 kHz, 5–10 s), diluted in electrophoresis sample buffer (50 % glycerol, 10 % SDS, 310 mM Tris–HCl, pH 6.8; 100 mM DTT, 0.02 % bromophenol blue) and boiled for 10 min. About 30 lg protein was separated by 7.5 % SDS-PAGE gel. Proteins were transferred electrophoretically to nitrocellulose membrane. Membranes were blocked with 5

% non-fat dry milk in PBS (30 min, room temperature), and incubated overnight at 4°C with anti-HMGB1 primary antibody (1:700; mouse monoclonal anti-HMGB1 antibody) and rabbit anti-actin primary antibody (1:1500). After washing with PBST (0.1 % Tween-20 in PBS) membranes were incubated with HRP-conjugated secondary antibodies (1:1000) HRP-conjugated goat antirabbit and anti-mouse IgG in PBS containing 5 % non-fat dry milk for 1 h. Luminescence signals were detected by enhanced chemiluminescence (ECL) reaction according to the manufacturer's instruction.

Experiments on mouse immortalized cell lines

Cell culturing

Murine C2C12 skeletal muscle cell line was obtained from the European Collection of Cell Cultures (ECACC). RAW 264.7 mouse macrophage cell line (American Type Culture Collection) was a kind gift of Prof. L. Virágh (Department of Medical Chemistry, University of Debrecen, Debrecen, Hungary). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM-high glucose, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin, 10% fetal bovine serum (FBS) for proliferation and 2% horse serum (HS) for myoblast differentiation and were kept at 37°C in an atmosphere containing 5% CO_2. Medium was changed to fresh in every second day.

Cell culturing protocol for examination of the proliferation of co-cultures

For monitoring the proliferation in the co-culture, 10^5 C2C12 cells in 200 µl medium were seeded on the marginal zone of a 3 cm petri dish, and 5×10^3 RAW264.7 cells in 100 µl medium were seeded on the center of the same dish. When cells were settled to the surface, 2 ml culture medium was added after a gentle rinse with phosphate buffered saline (PBS). Hence, the two cell types were cultured together without direct cellcell contact for a while. Monocultures were seeded in the same manner for accurate comparison. Medium was changed every second day. Cells were stained with May-Grünwald and Giemsa solution according to the protocol. At the bottom of the plastic cell-culture plate C2C12 cells were washed 3 times with PBS, fixed in methanol for 5 min and stained with Giemsa solution (Molar Chemicals Kft, Halásztelek, Hungary) for 5 min. Next, May-Grünwald (25× dilution in tap water) solution was added to the cells. 15 min later cells were washed in tap water and let dried. Photos were taken by a cell imaging system and were analyzed with ImageJ software to determine the area covered by cells.

Cell culturing protocol for monitoring the IL-6 production of co-cultures

5×10^2 C2C12 cells and 50 RAW264.7 macrophages were mixed and seeded on a 96-well-plate in 200 µl proliferating medium. On day 3 growth medium was exchanged to one supplemented with 2% HS to facilitate myoblast differentiation. On day 1, 3, 5, 7, and 9 culture medium were collected, centrifuged (2000 rpm, 5 min) and stored at −80°C until further use. For proper comparison, C2C12 and RAW 264.7 cell monocultures were seeded and handled in the same manner.

Cell culturing and pravastatin treatment protocol for IL-6 cytokine assay

10^5 C2C12 cells were seeded on 24-well-plates in 2 ml growth medium and on day 3 the medium was changed to medium extended with 2% HS to start myoblast differentiation. On day 6, when cultures contained considerable amount of myotubes, 5×104 RAW 264.7 macrophage cells were seeded on muscle cells, and the co-culture was treated with 500 µM pravastatin for 24 h in serum-free DMEM. Culture medium was collected, centrifuged, and stored at −80°C until use. For proper comparison monocultures were cultured and handled in the same manner. Non-treated cells were used as controls.

Cell culturing and pravastatin treatment protocol for the examination of cell proliferation

10^3 C2C12 cells and 5×10^2 RAW 264.7 macrophages were seeded on 96-well-plates in monocultures. After cells settled to the bottom of the plate, 500 µM pravastatin was added to the wells in growth medium containing 10% FBS. after 24 h, photos of the wells were taken, and the area covered by cells was determined by ImageJ software.

Cell culturing and pravastatin treatment protocol for the examination of C2C12 cells differentiation

5×10^2 C2C12 cells were seeded on 96-well-plates in 10% FBS containing growth medium. On day 3 medium was changed to low serum containing differentiating medium, and on day 4 pravastatin (500 µM) was added to the cells. On day 5, 7, and 9 treated and non-treated control cells were washed carefully in PBS and stained according to May-Grünwald Giemsa. Starting at day 4 culture medium was changed in every second day to fresh medium with or without pravastatin (treated and non-treated cultures, respectively). Photos of the cultures were taken, and the number of myotubes and the stained nuclei were counted.

“Cross-feeding” experiment on cell cultures

On the 5th day after seeding RAW 264.7 or C2C12 cells on 96-well-plates, culture medium was collected from monocultures, centrifuged (2000 rpm, 5 min), the supernatant was filtered (0.22 μm sterile syringe filter, Sigma-Aldrich). Half the volume of C2C12 cell culture medium supplemented with equal amount of fresh medium was added onto RAW 264.7 macrophages, and vice versa. After 24-h culturing, on day 6, the culture medium was collected again, centrifuged, filtered as previously described and used for ELISA measurement. Culture medium from parallel co-culture without cross-feeding was used as control.

IL-6 cytokine measurement by ELISA

Concentration of IL-6 in cell culture medium was determined by commercially available ELISA kit according to the manufacturer’s instructions. For testing we used 3 biological replicates.

IL-6 fluorescent staining

Co-cultured cells were treated with 3 μg/ml Brefeldin-A (Sigma-Aldrich) for 1 h to inhibit the protein transport from the endoplasmic reticulum to the Golgi apparatus. Cells were washed three times in PBS and fixed in 4% paraformaldehyde (PFA) (pH 7.4) for 15 min at room temperature. After quenching formaldehyde crosslinking reaction with glycine (30 mM glycine in PBS, pH 7.4, 5 min) cells were permeabilized with 0.5% TritonX-100 in PBS for 20 min. Non-specific binding sites were blocked for 1 h, then with primary antibody against IL-6 overnight at 4°C. Cells were washed three times in 0.1% Tween-20 in PBS and incubated with secondary antibody for 1 h at room temperature. After washing three times in PBS cells were covered by Mowiol 4-88 and glass coverslips. Only positivity of staining was evaluated without quantitative measurement of fluorescence intensity.

Measurement of cell viability using MTT-assay

On day 7 of culturing we determined the viability of control and pravastatin treated C2C12 cells by MTT assay. Briefly, in metabolically active cells mitochondrial dehydrogenases reduce the yellow MTT to bluish formazan product. Cells were incubated with 0.01% MTT reagent for 30 min at 37°C in an atmosphere containing 5% CO_2. After incubation, culture medium was discarded, and cells were lysed in 100 μl DMSO. Solubilized cells were transferred to a clear ELISA plate and optical density was measured at 550 nm. The absorbance obtained from pravastatin-treated cells was expressed as a percentage of that obtained from untreated control cells.

Statistical analysis

Descriptive statistics and statistical test results were calculated using SigmaPlot 12 with integrated SigmaStat (Systat Software Inc., Chicago, IL, USA). Mann–Whitney Rank Sum Test was used for comparison of two groups of non-normally distributed data. When three such groups were compared, Kruskal–Wallis one way analysis of variance on ranks (ANOVA on Ranks) was used in conjunction with Dunn's method for post hoc pairwise testing. Nonlinear curve fits were calculated and compared using GraphPad Prism. Results are presented as mean ± SEM (standard error of the mean). Differences were regarded significant when *p* did not exceed 0.05. For studies in mouse cell lines, all data are representative of at least three independent experiments. Averages are expressed as mean ± standard deviation (SD). Differences were assessed using one-way analysis of variance (ANOVA) and all pair wise multiple comparison procedures (Bonferroni multiple comparison). In some cases unpaired T-test was used.

Results

Results of studies on human striated muscle tissue

Decreased cell proliferation capacity in primary cell cultures initiated from muscle samples from patients with idiopathic inflammatory myopathy

Primary cell cultures of human skeletal muscle satellite cells derived from IIM patients without corticosteroid treatment (n = 6) were compared to cell cultures derived from IIM patients already receiving corticosteroid treatment (n = 3) and cell cultures derived from patients suffering from coxarthrosis (n = 7) to provide a reference base. Myogenic nuclei counts were normalized to the number of such nuclei on the 2nd day of culturing to correct for any difference caused by the uneven cell numbers in the initial preparation. Two-parameter exponential growth curves ($y = y_0 * \exp(k * x)$) were fitted. Nonlinear fitting and curve comparison showed that the three datasets are best fitted with three individual curves ($p<0.0001$). The k parameter values showing the growth rate were found to be 0.307 ± 0.028, 0.211 ± 0.035, and 0.295 ± 0.043 for coxarthrotic, untreated myositis patients, and corticosteroid- treated myositis patients, respectively. These values clearly indicate that the number of myogenic nuclei (nuclei in satellite cells, myoblasts and myotubes) shows a lower rate of increase in cultures derived from untreated IIM patients when compared to the findings in coxarthrotic primary cultures. Standard corticosteroid treatment of IIM patients increases the growth rate of primary muscle cell cultures to the coxarthrotic level. These effects were observed throughout a 11 day period of culturing. Typical images used for these calculations are shown for all groups and two different time points. In contrast with the proliferation rate, the fusion capacity of precursor cells to adult skeletal muscle fibers is not impaired in primary

cultures from patients with a confirmed diagnosis of IIM. The 3-parameter sigmoidal curves ($y = a/(1 + \exp(-(x-x0)/b))$) fitted to the fusion index data are not significantly different from each other ($p = 0.285$). The curve for the cultures from untreated IIM patients is, however, somewhat shifted to the right: x_0 = 2.745 ± 0.25 and 3.404 ± 0.086 days (coxarthrosis and untreated IIM, respectively), reflecting a slight delay in cell fusion. In addition, treatment with corticosteroids elicits a shift to the left (x0 = 1.831 ± 0.29 days) similar in size to the effect of untreated myositis compared to the coxarthrotic curve. Phase contrast micrographs clearly demonstrate that the morphology of skeletal muscle cells is not altered in an advanced stage of proliferation and early stage of differentiation, on the 6th day of culturing. At this time, myotubes contain 2–10 nuclei in both coxarthrotic cultures and cultures from IIM patients, but the lower number of cells and nuclei are clearly evident on the images the 10th day of culturing, these differences are even more pronounced.

Muscle samples from patients with idiopathic inflammatory myopathy show less regenerating muscle fiber and HMGB1 localized in the nucleus

Histological sections of adult muscle strips were stained for HMGB1in patients with coxarthrosis (n = 4) or with IIM without and with corticosteroid treatment (n = 4 in both groups). Samples from some patients were used in both the cell culturing and immunohistochemical studies, while others were used only in one kind of study. Immunohistochemical studies revealed less HMGB1 positive myogenic nuclei in the muscle fibres of IIM patients than in the coxathrotic group. The infiltrating mononuclear cells and endothelial cells showed HMGB1 positivity in all groups. The nuclei of the skeletal muscle fibres were either HMGB1+ or HMGB1- in all groups. Regions with mononuclear cell infiltration were also found in the muscles of coxarthrotic patients but the inflammatory infiltration was more prominent in the samples from patients with IIM without corticosteroid treatment. The number of HMGB1+ myogenic nuclei per cross sectional area was significantly ($p<0.05$) lower in both the untreated (402.5 ± 60.2 mm-2) the treated (360.1 ± 77.5 mm-2) IIM groups than in the coxarthrotic group (882.74 ± 155.1 mm-2). In order to identify signs of muscle regeneration the ratio of myotubes with central nuclei was investigated. Centralized nuclei were counted in transversal sections of skeletal muscle samples obtained from coxarthrotic patients (n = 4), patients suffering from IIM and not yet receiving corticosteroid treatment (n = 4) and patients suffering from IIM and receiving methylprednisolon treatment (n = 3). An average of six regions of interests were screened for centralized nuclei within the cross-section of the skeletal muscle fibre and the ratio of such nuclei was determined in proportion to the total number of myogenic nuclei. We found that in samples from coxarthrotic patients 4.53 ± 1.35 % of all myogenic nuclei were central, while in IIM patients before corticosteroid treatment the percentage decreased to 1.37 ± 0.51 %, which is a significant reduction ($p = 0.039$). The ratio was 2.32 ± 0.62 % in IIM

patients already receiving corticosteroid treatment, which does not differ significantly from either of the two other groups.

Muscle samples from patients with idiopathic inflammatory myopathy contain less HMGB1 than controls

Western blots were performed to determine the level of HMGB1 protein in samples from coxarthrotic patients (n = 8), IIM patients without corticosteroid treatment (n = 9) and IIM patients with corticosteroid treatment (n = 6). Five samples were excluded later due to ineligible diagnoses (e.g. systemic lupus erythematosus) provided by the pathologist. For the 23 remaining samples the average relative optical densities (normalized to the level of actin) per group were 0.488 ± 0.15, 0.157 ± 0.033, and 0.14 ± 0.039 (coxarthrosis, myositis untreated, and myositis treated; respectively). The decrease of HMGB1 levels between the coxarthrotic group and the two groups of IIM patients proved to be significant ($p<0.05$), but the difference between the treated and untreated myositis groups was not significant.

Results of experiments on mouse cell lines

Proliferation was not affected by co-culturing of RAW 264.7 macrophages and C2C12 muscle cells

To study the effects of two cell types on each other, proliferation of C2C12 myoblasts and RAW 264.7 macrophages was examined in mono- and co-culture. One day after seeding the area covered by C2C12 cells was 2.3 ± 0.9% in monoculture and 3.1 ± 1.2% in co-culture ($p > 0.05$), and in case of RAW 264.7 cells it was 8.0 ± 1.9% in monoculture and 5.8 ± 2.0% in co-culture ($p > 0.05$), respectively. On day 3 the difference between the two cell types was more prominent, the area covered by C2C12 cells was 10.1 ± 3.4% in monoculture and 12.1 ± 5.4% in co-culture ($p > 0.05$) and in case of RAW 264.7 cells it was 34.6 ± 6.6% in monoculture and 31.6 ± 3.6% in co-culture ($p > 0.05$). Rate of RAW 264.7 cells proliferation was significantly higher than C2C12 cells from day 3 of culturing both in monoculture ($p < 0.0001$) and co-culture ($p < 0.0001$). On the other hand, co-culturing had no effect on the rate of cell division neither for myoblasts or for macrophages ($p > 0.05$).

IL-6 production is enhanced in co-culture of C2C12 and RAW 264.7 cells

By using immunostaining method to detect IL-6 production in co-culture of C2C12 and RAW 264.7 cells, IL-6 production was found in both myogenic cells and monocytes/macrophages in the proliferation and differentiation phase of myogenesis. Monitoring the IL-6 content in the culture medium showed low level of IL-6 release without significant difference between the monocultures and coculture during proliferation phase of myogenesis (1–3 days)

of culturing. On day 5 IL-6 concentration in cocultures started to elevate gradually while it remained low in both monocultures. The amount of released IL-6 increased even further and was significantly higher compared to monocultures on day 7 and 9. Even though both C2C12 and RAW 264.7 cells can release measurable amount of IL-6, its level remained low in the culture medium of monocultures during the 9-day culturing period.

Pravastatin enhances IL-6 production of co-culture

Since statins have been shown to reduce inflammation, we hypothesized that the water-soluble pravastatin would decrease IL-6 level in co-cultures. To test this hypothesis, we treated 6-day-old monocultures and co-cultures with 500 μM pravastatin for 24 h. However, in contrast to our hypothesis, we observed that pravastatin significantly increased IL-6 production in C2C12 monocultures (control 2.3 ± 0.2 pg/ml; pravastatin treated 18.0 ± 1.5 pg/ml, $p < 0.001$) and in co-cultures as well (control 129.3 ± 5.8 pg/ ml; pravastatin 945.8 ± 50.2 pg/ml; $p < 0.001$) and did not modify considerably the IL-6 production of RAW 264.7 monocultures (control 2.5 ± 0.1 pg/ml; pravastatin 1.8 ± 0.1 pg/ml; $p > 0.05$).

Pravastatin significantly impairs the proliferative ability of C2C12 muscle cells

We then hypothesized that increased IL-6 production of coculture might originate from the increased number of cells due to pravastatin treatment. Thus, we examined the proliferation of 24-hour-old monocultures treated with pravastatin. Number of myoblasts decreased significantly after 24 h pravastatin treatment ($p < 0.01$). In contrast, to that number of macrophages was unchanged after 24 h treatment ($p > 0.05$). Thus, it is unlikely that the elevated IL-6 level in the co-culture after pravastatin treatment is the result of enhanced proliferation capacity of cells.

Pravastatin impairs C2C12 differentiation

Since pravastatin has a considerable negative effect on the proliferation of C2C12 cells, we studied the effects of pravas tatin on differentiation, too. Fusion index (FI) represents the ratio of nuclei in myotubes to all nuclei in the cell culture. Differentiating C2C12 cells were treated with pravastatin for 9 days, and we detected significantly lower number of myotubes ($p < 0.05$) on the 7th and 9th days of culturing than in untreated cultures. While fusion index of control cells showed gradual increase from day 5 to 9, treated values has not been changed at all. However, pravastatin treated cultures had similar ($p > 0.3$) viability to those under control conditions.

Summary

Statins are the first line drugs for treating hypercholesterolemia, but rarely, they can have severe, muscle-damaging side effects.
We performed experiments on human surgical and biopsy speciments, primary muscle cell cultures, and mouse cell lines.
Muscle inflammation was modeled in vitro on cocultures of C2C12 and RAW264.7 cells.
The number of regenerating fibers was lower in the muscles derived from patients without steroid treatment, and in the primary cell cultures initiated from their samples, the rate of proliferation was slower compared to the controls. Previous corticosteroid treatment reduced the inflammatory infiltration in skeletal muscle. HMGB1 was found in the nuclei of muscle cells and infiltrating cells regardless of disease or steroid treatment. Despite pre-treatment, samples derived from patients with myositis contained less HMGB1. Corticosteroid treatment had no effect either on total HMGB1 content of muscles or the number of HMGB1 positive nuclei or regenerating fibers. In cocultures cells did not affect on each other's proliferative capacity, however coexistence increased the IL-6 production of the coculture. At the stage of differentiation, IL-6 production of muscle cells was elevated even without the presence of macrophages, but the cytokine level was not as high as in a coculture of the same age. Pravastatin treatment further increased IL-6 production of muscle monocultures and cocultures and impaired their proliferation and differentiation ability. Pravastatin did not affect on IL-6 production or proliferation of macrophages.
Our results suggest that pravastatin may increase myositis in some cases and that HMGB1 may play a role in this process.

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Registry number: DEENK/6/2022.PL
Subject: PhD Publication List

Candidate: Karolina Cseri
Doctoral School: Doctoral School of Molecular Medicine
MTMT ID: 10055321

List of publications related to the dissertation

1. **Cseri, K.**, Szentesi, P., Csernoch, L.: IL-6 production of C2C12 cells is enhanced in the presence of macrophages and pravastatin.
   Gen. Physiol. Biophys. 40 (4), 307-315, 2021.
   DOI: http://dx.doi.org/10.4149/gpb_2021017
   IF: 1.512 (2020)

2. **Cseri, K.**, Vincze, J., Cseri, J., Fodor, J., Csernátony, Z., Csernoch, L., Dankó, K.: HMGB1 expression and muscle regeneration in idiopathic inflammatory myopathies and degenerative joint diseases.
   J. Muscle Res. Cell Motil. 36 (3), 255-262, 2015.
   DOI: http://dx.doi.org/10.1007/s10974-015-9411-7
   IF: 2.071

List of other publications

3. Csóka, B., Törő, G., Vindeirinho, J., Varga, Z. V., Koscsó, B., Németh, Z. H., Kókai, E., Antonioli, L., Suleiman, M., Marchetti, P., **Cseri, K.**, Deák, Á., Virág, L., Pacher, P., Bai, P., Haskó, G.: A2A adenosine receptors control pancreatic dysfunction in high-fat-diet induced obesity.
   Faseb J. 31 (11), 4985-4997, 2017.
   DOI: http://dx.doi.org/10.1096/fj.201700398R
   IF: 5.595

4. Pellegrini, C., Fornai, M., Colucci, R., Tirotta, E., Blandini, F., Levandis, G., Cerri, S., Segnani, C., Ippolito, C., Bernardini, N., **Cseri, K.**, Blandizzi, C., Haskó, G., Antonioli, L.: Alteration of colonic excitatory tachykininergic motility and enteric inflammation following dopaminergic nigrostriatal neurodegeneration.
   J Neuroinflammation. 13 (1), 146-159, 2016.
   DOI: http://dx.doi.org/10.1186/s12974-016-0608-5
   IF: 5.102

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5. Csóka, B., Németh, Z. H., Törő, G., Idzko, M., Zech, A., Koscsó, B., Spolarics, Z., Antonioli, L., **Cseri, K.**, Erdélyi, K., Pacher, P., Haskó, G.: Extracellular ATP protects against sepsis through macrophage P2X7 purinergic receptors by enhancing intracellular bacterial killing. *Faseb J. 29* (9), 3626-3637, 2015.
DOI: http://dx.doi.org/10.1096/fj.15-272450
IF: 5.299

6. Töröcsik, D., Kovács, D., Camera, E., Lovászi, M., **Cseri, K.**, Nagy, G. G., Molinaro, R., Rühl, R., Tax, G., Szabó, K., Picardo, M., Kemény, L., Zouboulis, C. C., Remenyik, É.: Leptin promotes proinflammatory lipid profile and induces inflammatory pathways in human SZ95 sebocytes. *Br. J. Dermatol. 171* (6), 1326-35, 2014.
IF: 4.275

7. Griger, Z., Nagy-Vincze, M., Bodoki, L., **Cseri, K.**, Hortobágyi, T., Dankó, K.: Nekrotizáló autoimmun myopathia esete. *Metabolizmus. 11* (5), 379-382, 2013.

8. Bodoki, L., Nagy-Vincze, M., Griger, Z., Csonka, T., **Cseri, K.**, Hortobágyi, T., Dankó, K.: Rituximabkezelés idiopathiás inflammatorikus myopathiákban. *LAM. 23* (1), 16-21, 2013.

9. Bodoki, L., Nagy-Vincze, M., Hortobágyi, T., Griger, Z., **Cseri, K.**, Szőllősi, L., Dankó, K.: Nekrotizáló autoimmun myopathia. *Orv. Hetil. 153* (38), 1502-1507, 2012.
DOI: http://dx.doi.org/10.1556/OH.2012.29450

Total IF of journals (all publications): 23,854
Total IF of journals (publications related to the dissertation): 3,583

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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