Regulatory properties of immunoglobulins on proliferation and apoptosis in murine muscle cells are dose-dependent

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Abstract

Intravenous immunoglobulins (IVIg) are widely used in idiopathic inflammatory muscle diseases. There are no in vitro or in vivo dose-finding studies for IVIg application under these pathological conditions. Therefore, we investigated the influence of Igs on proliferation and susceptibility to apoptosis in murine muscle cells derived from mice selected for high body weight (DU-6). Apoptosis was induced by serum deprivation at day 4 of cultivation, and cell proliferation and the proportions of apoptotic cells were determined at days 5 and 6. In summary, the effect of Ig on muscle cell growth was dependent on time of cultivation, on growth conditions (10%, 1% FBS) and on the applied doses of Ig. During exponential growth in growth medium (day 5, 10% FBS) almost all Ig doses from 1 to 20 mg/ml without and plus maltose decreased proliferation rate. This was not associated with decreases in apoptosis rate, except at 4 mg/ml Ig. In contrast, at day 5 in maintenance medium (1% FBS) and at day 6 under both growth conditions (1% and 10% FBS) increased proliferation rates were observed with 10 and 20 mg/ml Ig without and plus maltose, which was associated in parts with higher apoptosis rates. Decreases in apoptotic cells were found at day 5 in growth and maintenance medium in response to 4 mg/ml Ig without and plus maltose. On day 6, apoptosis remained unchanged with 4 mg/ml Ig without maltose, but it was increased when maltose was included. 4 mg/ml Ig approximates the dose applied under pathological conditions like polymyositis (PM) or dermatomyositis (DM) in men.

Our findings suggest that, besides immunomodulatory properties, IVIg exhibit regulatory functions for apoptosis and proliferation in muscle cells.

Key words: muscle cells, apoptosis, development, differentiation, proliferation, immunoglobulins

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Introduction

Dermatomyositis (DM), polymyositis (PM) and inclusion body myositis (IBM) are the three major forms of idiopathic inflammatory myopathies with distinct clinical, histological and pathogenetical features. Autoantibodies against myosin and nuclear molecules are found in the serum of PM and DM patients, and infiltration of lymphocytes and macrophages beneath the basal membrane of muscle fibres has been observed [7]. In addition, deposition of C5b-C9 membrane attack complex (MAC) in intramuscular capillaries has been reported in DM patients, which may lead to the destruction of endothelial cells followed by the development of microinfarctions in the muscle fascicles and by perifascular atrophy [12]. These immunological features are thought to act synergistically and eventually cause progression of muscle degeneration. To date, the role of classical apoptotic cell death of muscle cells in inflammatory myopathies remains an unresolved issue.

Susceptibility to induction of apoptosis appears to be a key property during development of mammalian skeletal muscle cells under physiological conditions [26]. The question, whether mature, postmitotic, multinucleated skeletal muscle cells can undergo apoptosis is discussed controversially [11, 27]. However, apoptotic cell death in skeletal muscle occurs under various pathologic conditions such as reperfusion after ischaemia and muscular dystrophy [13]. All features of apoptosis could not be detected in inflammatory muscle diseases [14]. Nevertheless, inflammatory cells displayed numerous DNA-fragmentation-positive nuclei and expression of apoptosis-related proteins indicating that apoptosis plays

a role in the regulation of the inflammatory response [23]. Furthermore, Sugiura et al. (1999) [22] could demonstrate that Fas and Fas ligand interaction induces apoptosis in inflammatory myopathies and cause directly muscle cell injury in PM.

Intravenous immunoglobulins (IVIg) are widely used in these pathological conditions. Several retrospective and uncontrolled studies have suggested that IVIg is beneficial especially in DM and PM [1]. Dalakas and co-workers [6] demonstrated the efficacy of IVIg in a prospective, double-blind, placebo-controlled study including repeated muscle biopsies in patients with DM. Beside a regulatory effect on the inflammatory response they could demonstrate an increase of muscle fibre diameter. Several mechanisms have been proposed for the therapeutical effect of IVIg [2, 15], e.g. inhibition of complement activities, neutralization of autoantibodies by anti-idiotypic antibodies, modulation of lymphocyte functions and cytokine synthesis as well as blockade of Fc-receptors on phagocytic cells.

Our previous reports comparing the susceptibility to apoptosis in different muscle cell lines during *in vitro* myogenesis could demonstrate that primary cell cultures derived from mice long-term selected for high body weight (DU-6) and cells from respective controls (DU-Ks) as well as C_2C_{12} muscle cells differ in *in vitro* DNA and protein accumulation [18, 19, 25, 30]. In addition, primary cultures (DU-6; DU-Ks) responded more sensitive to serum deprivation than C_2C_{12} cells as indicated by increased cell loss [18, 30], which may result from the closer distance of the primary muscle cells to the *in vivo* status.

The aim of the current study is to investigate the influence of different Ig concentrations on proliferation and susceptibility to apoptosis by serum deprivation in murine DU-6 muscle cells at different developmental stages representing cell proliferation and early cell differentiation. Attention is also paid to effects of sugar additives, since they are regularly used to stabilize IVIg preparations [28].

Materials and Methods

Animals and cell culture

A mouse line long-term selected over 107 generations for a high 6-weeks body weight (DU-6) was used in this study. Details of the selection procedure were described earlier [3, 4, 5]. The line is derived from the outbred strain Fzt:DU, which was obtained by systematic crossbreeding of four inbred with four outbred lines [21]. The animals were kept in a semi-barrier system with food and water available *ad libitum*. The selection line was created by allocating full sibs to this line. One female and one male were randomly picked at birth from 36 litters in generation 107. Cells were isolated from leg muscles according to a protocol given by Harper et al. [10]. Myogenic cells were enriched to 91.5% by differential pre-plating and Percoll density gradient (100%, 90%, 40%) centrifugation as verified by immunocytochemical desmin expression according to a modified protocol given by Yablonka-Reuveni et al. [31]. For experiments cells of the first passage were cultivated in DMEM (Dulbecco's modification of Eagle's medium, Life Technologies, Eggenstein, Germany; lot 3032314) supplemented with 0.02 M glutamine (Serva, Heidelberg, Germany), 100 IU/ml penicillin, 100µg/ml streptomycin and 2.5 µg/ml fungizone (Sigma-Aldrich, Deisenhofen, Germany) and foetal bovine serum (FBS; Life Technologies; lot 40G4992K) at 10% or 1% (v/v), respectively. All incubations were performed at 37°C under a humified atmosphere of 6% CO_2 in air. More details on animals, cell isolation and culture conditions are described elsewhere [19].

Experimental design

Cells were seeded at 7.8 x 10^3 cells/cm² in a volume of 100 µl in 96-well Primaria microtiter plates or in a volume of 1.5 ml in 35 mm plastic dishes (Falcon, Becton Dickinson, Heidelberg, Germany) and grown in DMEM with 10% FBS. At day 4, when the cultures were almost confluent, the medium was changed by different test media containing DMEM with 10% FBS, and 10% FBS plus 2, 4, and 20 mg/ml, respectively, of human immunoglobulin G (IgG) (IVIg, octagam; Octapharma, Langenfeld, Germany, containing 50 mg/ml IgG plus100 mg/ml maltose) or 1% FBS, and 1% FBS plus 2, 4, and 20 mg/ml IVIg, respectively, and continued for two days.

In additional experiments a pure IgG without maltose (Octapharma) was used at the same concentrations plus concentrations of 1 mg/ml and 10 mg/ml. Samples were taken daily from days 4 to 6 to determine the frequency of apoptotic cells by TUNEL assay as well as DNA synthesis rate by BrdU incorporation (days 5 and 6 only). Three independent experiments with three replicates for each day of cultivation at each test medium were performed for the analysis of apoptosis. Two independent experiments with eight replicates were performed for the BrdU test.

Analysis of cell proliferation

To determine the DNA synthesis rate, a colorimetric 5-bromo-2-desoxyuridine (BrdU) assay kit (Roche, Mannheim, Germany) was used. The suitability of this test for mouse muscle cells has been demonstrated elsewhere (Wittstock et al., 2001).

Assessment of apoptosis by tailing assay (TUNEL) and FACS analysis

The assessment of apoptosis was carried out by tailing assay (TUNEL assay) with flow cytometric analysis. Cells were trypsinized, fixed with 4% (w/v) paraformaldehyde, and permeabilized with 0.025% (v/v) NP-40 (Roche, Mannheim, Germany) as described previously [9]. Briefly, the cells were incubated in 50 µl

of a reaction mixture containing 10 µl tailing buffer (Roche), 0.15 nM fluorescein-12-ddUTP (Boehringer, Mannheim, Germany), 1 nM of dCTP, (Sigma-Aldrich) and 5 units terminal transferase (Roche). Reactions were allowed to proceed for 2 h under light protection at 37°C on a rotation shaker (400 rpm). Cells were then analysed by flow cytometry using EPICS XL (Coulter, Hialeah, FL, USA) to determine the percentage of fluorescein positive apoptotic cells. Omission of terminal transferase from the reaction mixture served as negative control and DNAse I (Roche) treatment as positive control.

Statistical Analysis

The data of the individual experiments with three to eight replicates each were subjected to analysis of variance using the GLM model of the SAS® system (release 8e, SAS Inst. Inc., Cary, NC, USA) with the fixed factors experiment, day of cultivation, and test medium. Least square means and standard errors (SE) are presented in the graphs. Individual differences between least square means were tested by Student's t-test. Significance was concluded for $p < 0.05$.

Results

Cell proliferation

Differences in cellular growth of muscle cells derived from mice selected for high body weight (DU-6) were determined by BrdU incorporation at days 5 and 6 of cultivation in medium with 10% FBS and 1% FBS after serum reduction at day 4 of cultivation, respectively. The influence of addition of different Ig concentrations was investigated, i.e. 1-20 mg/ml pure Ig (Figure 1) and Ig plus maltose 2-20 mg/ml (Figure 2) were applied.

Figure 1

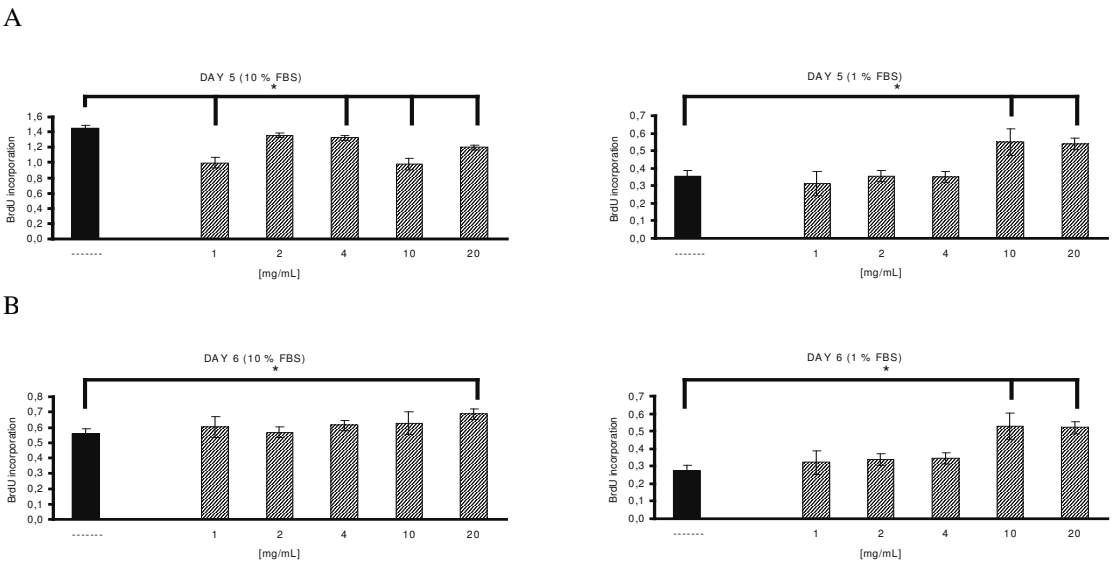

***Figure 1**. Proliferation of muscle cells derived from mice selected for high body weight (DU-6) as measured by BrdU incorporation ($A_{460\ nm} - A_{690\ nm}$) at days 5 (Fig. 5 A) and 6 (Fig. 5 B) of cultivation under growth conditions with 10% FBS and under maintenance conditions with 1% FBS. Serum was reduced to 1% on day 4 of cultivation. Cultivation was done without Ig (black column, n = 12) and with 1 (n = 3), 2 (n = 12), 4 (n = 12), 10 (n = 3) and 20 (n = 12) mg/ml Ig, respectively. Means and SE are represented as columns and error bars, respectively. Significance to control (0 mg/ml Ig) is indicated by * $p < 0.05$.*

At day 5 there was a markedly lower proliferation rate at cultivation with 10% FBS, when Ig was added at concentrations of 1, 4, 10 and 20 mg/ml ($p < 0.05$), whereas this effect was smaller in presence of 2 mg/ml ($p > 0.05$) Ig. After serum reduction to 1% at day 4, there were no significant changes in proliferation at day 5 of cultivation in response to 1, 2 and 4 mg/ml Ig. However, an increase could be observed in response to cultivation with 10 and 20 mg/ml Ig ($p < 0.05$). At day 6, myoblasts in 10% FBS cultures showed basically a lower proliferation than at day 5 ($p < 0.05$), but an increase in proliferation over untreated cultures only after using an Ig concentration of 20 mg/ml ($p < 0.05$). In 1% FBS cultures increases in proliferation were found in the presence of 10 and 20 mg/ml Ig ($p < 0.05$) (Figure 1), which is consistent with the results of day 5 of cultivation.

When Ig was administered together with maltose, the 10% FBS cultures showed a lower proliferation on day 5 of cultivation for all concentrations of Ig (2, 4, 20 mg /ml plus maltose) compared to cultivation without Ig ($p < 0.05$) (Figure 2). In 1% FBS cultures proliferation was higher in response to 20 mg/ml Ig plus maltose ($p < 0.05$), but remained unchanged after application of 2 and 4 mg/ml Ig plus maltose. At day 6, the addition of 20 mg/ml Ig plus maltose induced a significantly higher proliferation ($p < 0.05$) in 10% FBS cultures, whereas in 1% FBS cultures significantly higher proliferation rates could be noted at concentrations of 4 and 20 mg/ml (Figure 2).

Figure 2

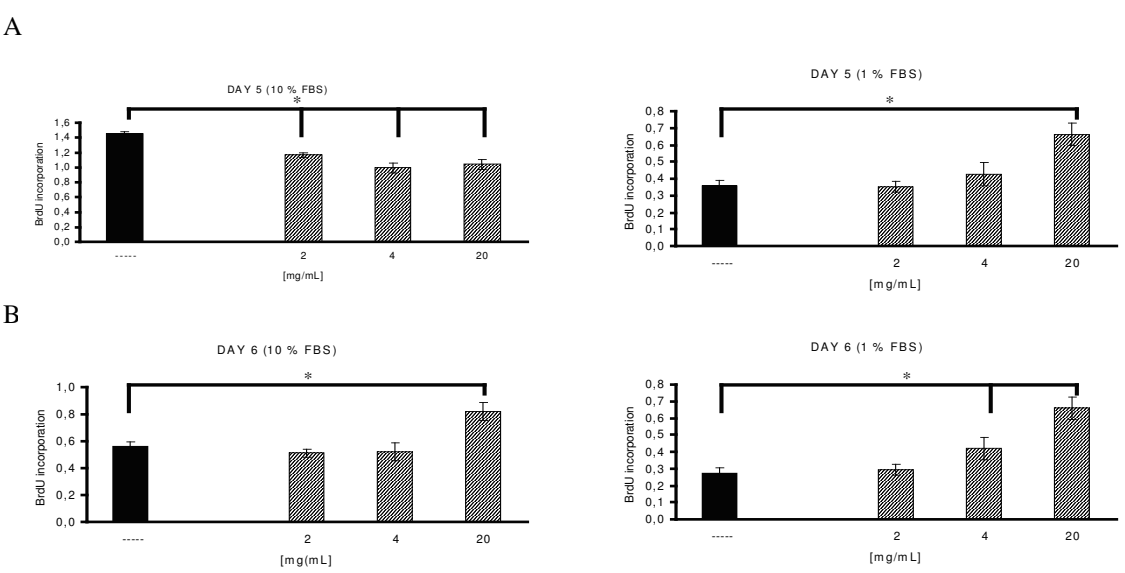

Figure 2. *Proliferation of muscle cells derived from mice selected for high body weight (DU-6) as measured by BrdU incorporation at days 5 (Fig. 5 A) and 6 (Fig. 5 B) of cultivation under growth conditions with 10% FBS and under maintenance conditions with 1% FBS. Serum was reduced to 1% on day 4 of cultivation. Cells were cultivated without Ig (black column, n = 12) and with 2 (n = 9), 4 (n = 3) and 20 (n = 3) mg/ml Ig plus maltose, respectively. Means and SE are represented as columns and error bars, respectively. Significance to control (0 mg/ml Ig) is indicated by * $p < 0.05$.*

With respect to time induced changes, at all growth conditions a decrease of proliferation occurred between days 5 and day 6 in 10% FBS ($p < 0.5$), but not in 1% FBS cultures . Additionally, serum reduction to 1% FBS at day 4 led to significantly lower proliferation rates. With Ig, especially at high concentrations, the decreases in proliferation by time and by serum reduction could not be prevented.

Apoptosis

To examine whether different Ig preparations had an influence on apoptosis of Du-6 cells in response to serum deprivation, the percentages of apoptotic cells were determined by TUNEL assay in parallel to proliferation. Measurements were done at days 5 and 6 of cultivation in 10% FBS and 1% FBS after serum reduction at day 4, respectively. Results are shown for cultivation after addition of pure Ig (1-20 mg/ml) and Ig plus maltose (2-20 mg/ml), respectively, (Figures 3 and 4).

At day 5 the apoptosis rate was significantly decreased after addition of 4 mg/ml Ig during growth in both 10% FBS and 1% FBS cultures. At day 6 in cultures with 10% FBS apoptosis was increased in response to 20 mg/ml Ig, whereas in cultures with 1% FBS 10 mg/ml Ig increased the percentage of apoptotic cells ($p< 0.05$) (Figure 3). After adding Ig plus maltose (Figure 4) there were no differences in apoptotic cell death from control on day 5 of cultivation in medium with 10% FBS. In 1% FBS cultures, however, both 2 and 4 mg/ml Ig plus maltose lowered, whereas 20 mg/ml Ig increased the percentage of apoptotic cells (Fig. 4) ($p < 0.05$). On day 6 of cultivation, both with 10% and with 1% FBS, the addition of 2 mg/ml Ig plus maltose did not change apoptosis rate, whereas increases were observed in response to 4 and 20 mg/ml Ig plus maltose ($p < 0.05$).

Basically, an increase of apoptosis was seen under all cultivation conditions from day 5 to day 6, which tended to be more pronounced in serum reduced cultures. Serum reduction from 10 to 1% FBS on day 4 led to

higher apoptosis rates at day 6, but not yet at day 5 in the control and at most concentrations of Ig except 20 mg/ml that showed already high apoptosis at 10% FBS.

Figure 3

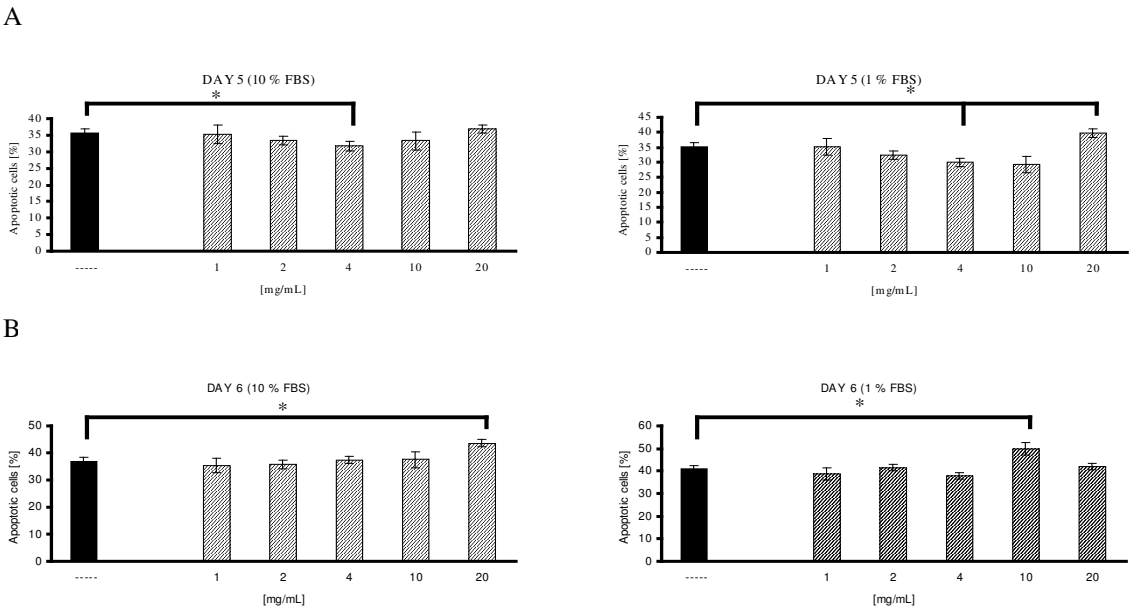

Figure 3. *Apoptosis of muscle cells derived from mice selected for high body weight (DU-6) as measured by TUNEL assay at days 5 (Fig. 5 A) and 6 (Fig. 5 B) of cultivation under growth conditions with 10% FBS and under maintenance conditions with 1% FBS Serum was reduced to 1% on day 4 of cultivation. Cells were cultivated without Ig (n = 12) and with 1 (n = 3), 2 (n = 12), 4 (n = 12), 10 (n = 3) and 20 (n = 12) mg/ml Ig, respectively. Results of cultivation without Ig are presented as black columns. Means and SE are presented in the graphs. Significance to control (0 mg/ml Ig) is indicated by * $p < 0.05$.*

In summary, the effect of Ig on muscle cell growth was dependent on time of cultivation, on growth conditions (10%, 1% FBS) and on the applied doses of Ig. During exponential growth in growth medium (day 5, 10% FBS) almost all Ig doses from 1 to 20 mg/ml without and plus maltose decreased proliferation rate. This was not associated with decreases in apoptosis rate, except at 4 mg/ml Ig. In contrast, at day 5 in maintenance medium (1% FBS) and at day 6 under both growth conditions (1% and 10% FBS) increased proliferation rates were observed with 10 and 20 mg/ml Ig without and plus maltose, which was associated in parts with higher apoptosis rates. Decreases in apoptotic cells were found at day 5 in growth and maintenance medium in response to 4 mg/ml Ig without and plus maltose. On day 6, apoptosis remained unchanged with 4 mg/ml Ig without maltose, but it was increased when maltose was included.

Discussion

The present study compares the extent of proliferation and apoptotic cell death of muscle cells derived from DU-6 mice selected for high body weight under the influence of Ig preparations used for treatment of inflammatory myopathies. The DU-6 cells were chosen for the experiments, because they have been previously found to provide a suitable model of muscle cell growth and differentiation , to be highly susceptible to apoptosis and to be easily cultivated at high cell numbers [30]. Cells were investigated from day 4, when the cultures became confluent, to day 6 of cultivation, when first myotubes were formed. The suitability of the methods used for assessment of proliferation (BrdU incorporation) and apoptosis (TUNEL assay) in our study has been proven in previous studies on the same muscle cells [18, 29, 30].

Our in vitro study demonstrates, that the effect of Ig on muscle cell growth was dependent on time of cultivation, on growth conditions (10%, 1% FBS) and on the applied doses of Ig. During exponential growth in growth medium (day 5, 10% FBS) almost all Ig doses from 1 to 20 mg/ml without and plus maltose decreased proliferation rate. This was not associated with decreases in apoptosis rate, except at 4 mg/ml Ig. In

contrast, at day 5 in maintenance medium (1% FBS) and at day 6 under both growth conditions (1% and 10% FBS) increased proliferation rates were observed with 10 and 20 mg/ml Ig without and plus maltose, which was associated in parts with higher apoptosis rates. Decreases in apoptotic cells were found at day 5 in growth and maintenance medium in response to 4 mg/ml Ig without and plus maltose. On day 6, apoptosis remained unchanged with 4 mg/ml Ig without maltose, but it was increased when maltose was included. This may be in accordance with findings from Williams and co-workers [28], who suggested that sugars used to stabilize IVIg preparations may cause cytotoxic side effects at therapeutically relevant doses of IVIg.

Figure 4

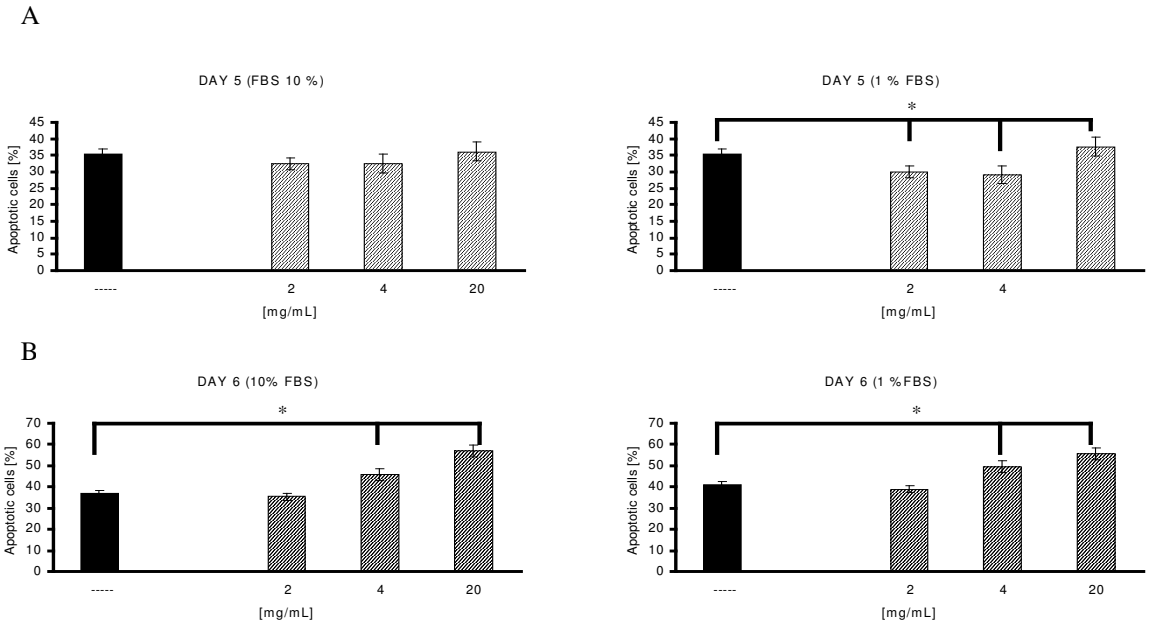

Figure 4. *Apoptosis of muscle cells derived from mice selected for high body weight (DU-6) as measured by TUNEL assay at days 5 (Fig. 5 A) and 6 (Fig. 5 B) of cultivation under growth conditions with 10% FBS and under maintenance conditions with 1% FBS after one and two days, respectively, of serum reduction without Ig (n = 12) and with 2 (n = 9), 4 (n = 3) and 20 (n = 3) mg/ml Ig plus maltose, resp.). Results of cultivation without Ig are presented as black columns. Means and SE are presented in the graphs. Significance to control (0 mg/ml Ig) is indicated by * $p < 0.05$.*

Usually IVIg is applied at a dose of 0.4 g/kg body weight in regular terms and of 2.0 g/kg body weight in an initial booster course. There are no dose-finding studies for treatment of myopathies so far [8]. In multiple sclerosis, there is experimental evidence that immunomodulatory properties of IVIg are dose-dependent [20]. Previous studies could demonstrate a beneficial effect of IVIg on inflammatory response in PM as well as in DM in man [1, 6]. In an animal model of experimental autoimmune myositis (EAM) and adopted EAM in SJL/J mice this findings could be confirmed by Wada et al. [24]. In their study IVIg has been applied at doses of 100, 200, 400 or 800 mg/kg body weight/day for five consecutive days and modulation of the inflammatory response was investigated immunohistochemically. The authors could demonstrate prevention of development of myositis by several mechanisms such as reduction of anti-myosin antibodies and blocking of complement activation. These effects of IVIg were dose-dependent with highest reduction of inflammatory and necrotic changes in skeletal muscle by approximately 400 mg IVIg/kg body weight/day, a dosage used for the treatment of DM and PM in men. Recently, Raju & Dalakas [16, 17] could demonstrate by microarray experiments and real-time PCR an IVIg induced muscle remodelling and regeneration in DM, but not IBM. IVIg is modulating a subset of clinically relevant immunoregulatory or structural genes associated with clinical improvement and restoration of muscle cytoarchitecture. Our findings suggest that, besides immunomodulatory properties, IVIg may exhibit regulatory functions in muscle cell growth by influencing proliferation and apoptosis.

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