Cathepsins Are Upregulated by IFN-γ/STAT1 in Human Muscle Culture: A Possible Active Factor in Dermatomyositis

Eduard Gallardo, PhD, Irene de Andrés, PhD, and Isabel Illa, MD

Abstract. The aim of this work was to study which genes upregulated by the IFN-γ/STAT1 system in human muscle might be involved in the process of muscle fiber atrophy in dermatomyositis (DM). These proteins included proteases (cathepsins B and L, calpain), proteins implicated in apoptosis and cell cycle (Bcl-x(l), Fas, p21), structural proteins (β-actin, utrophin, desmin), and other proteins whose expression is known to be modified by IFN-γ (neural cell adhesion molecule (N-CAM), major histocompatibility complex-I (MHC-I)). We performed immunocytochemistry, Western blot, and semiquantitative reverse transcriptase-polymerase chain reaction using human muscle cultures. We found upregulation of cathepsins B and L, bcl-x(l) and p21 while N-CAM, calpain, utrophin, desmin, β-actin and Fas remained at basal levels. Immunohistochemistry on frozen sections from biopsies of patients with different muscle diseases showed upregulation of cathepsin L and calpain in perifascicular muscle fibers in DM. In view of these results, the increased expression of cathepsins L and B after IFN-γ stimulation in muscle cultures and its inhibition using fludarabine, a STAT1 blocker, further support our previous studies and suggest that the increased expression of cathepsins detected in perifascicular muscle fibers in DM is mediated by IFN-γ/STAT1 and contributes to their atrophy.

Key Words: Cathepsins; Dermatomyositis; Fludarabine; IFN-γ/STAT1; Inflammatory myopathy.

INTRODUCTION

Dermatomyositis (DM) belongs to a heterogeneous group of disorders identified as inflammatory myopathies (IM), characterized by proximal weakness and the presence of inflammatory infiltrates in the muscle biopsy. A humorally mediated immune attack could promote focal angiopathy, which due to endofascicular hypoperfusion, would eventually lead to perifascicular atrophy, a characteristic of DM (1–4).

Several studies have been performed to elucidate the role of cytokine expression in the pathogenesis of IM; however, some contradictory findings have been reported, as reviewed by Lundberg and Nyberg (5). For instance, IL-1α was found to be abnormally expressed in muscle fibers (6), whereas others found it only in inflammatory cells (7, 8). Furthermore, expression of TNF-α was detected in the muscle fibers of patients with DM (7), while other authors reported its expression only in inflammatory cells (9). Interestingly, in another study, expression of TNF-α was found in many endothelial cells and upregulation of TNF-R75 in perifascicular and perimysial endothelia in DM, suggesting a role for TNF-α in endothelial cell degeneration in this inflammatory myopathy (10). Additionally, using immunohistochemical analysis, IL-4 has been detected in all patients with DM (7), whereas other authors found that its mRNA expression in DM and IBM was weak and inconsistent (11).

In this paper we have focused on the role of IFN-γ in IM. Previous studies found IFN-γ expression in the muscle biopsies of 2 of 5 patients with DM by reverse transcriptase-polymerase chain reaction (RT-PCR) (11), and another found that the biopsies of 6 of 10 DM patients presented positive inflammatory cells for IFN-γ by immunohistochemistry (7). These authors suggest some explanations for the restricted expression of this and other cytokines in IM: a) Either IFN-γ is rapidly degraded or absorbed by specific inhibitors upon release, or b) the cases studied have already reached an active inflammatory state so that proinflammatory cytokines have already been downregulated. To address these possibilities, we studied the expression of STAT1, a transcription factor activated by IFN-γ, in IM.

We observed increased expression of the signal transducer and activator of transcription 1 (STAT1) (a molecule known to be activated in cell response to IFN-γ [12]) in the perifascicular muscle fibers of patients with DM (13). In contrast, in other inflammatory myopathies, only infiltrating cells in muscle stained positive for STAT1. The same study also found that IFN-γ was able to activate STAT1 in human muscle culture, which in turn activated other genes, suggesting that these effects might also be involved in perifascicular muscle fiber alterations in DM.

Phosphorylated STAT1 forms homodimers that translocate into the nucleus, where they are able to bind to defined DNA sequences (TTNCNNNA, GAS for gamma activated site) and initiate the transcription of genes bearing this sequence in their promoters (14). Over 200 genes are now known to be regulated by IFN-γ (15),...
although only some of them are known to be activated through STAT1. Among these are the genes involved in many different cellular processes in various cell types. In this study we examined the expression of some of the genes involved in muscle fiber damage, regeneration, and apoptosis in human muscle culture and their possible pathophysiological role in muscle fiber damage in DM. Proteases were specifically chosen for the following reasons: 1) activation of endogenous, lysosomal and extralysosomal proteases may be an important mechanism of muscle fiber destruction in myopathies (16); 2) immunocytochemical studies have shown that all atrophic perifascicular muscle fibers in DM stained strongly for different proteases (17); and 3) some proteases are known to be upregulated by IFN-γ.

We also studied the effects of fludarabine, a nucleoside analog that partially blocks STAT1 (18), to determine whether the effects of IFN-γ are indeed mediated by this transcription factor.

**MATERIALS AND METHODS**

**Human Muscle Culture**

We studied frozen sections from the biopsies of 5 patients with DM, 5 patients with polymyositis (PM), and 5 controls with normal muscle biopsy using the same immunohistochemical procedure. Patients were biopsied prior to treatment except for 1 patient with polymyositis (PM) who was taking cyclosporine. Patients had a mean age of 48 yr for both DM (34–60 yr) and PM (37–59 yr), and a duration of symptoms from 3 months to 20 yr, and 6 months to 3 yr for DM and PM, respectively.

Human muscle biopsies were cut into small explants and cultured in Eagles minimum essential medium (Mediatech, Reston, VA) with 10% heat inactivated fetal calf serum (MA Bioproducts, Springville, MD), 2% chick embryo extract (GIBCO, BRL, Bethesda, MD), 50 mM glutamine, and gentamycin according to standard technique (19). Culture medium was changed twice a week and cells were examined to assess the development of monolayers of myoblasts or confluent myotubes. The experiments were performed when the percentage of myoblasts/myotubes positive for NCAM and the number of nuclei forming part of myotubes were about 90% (20).

**Culture Stimulation**

Human muscle cultures were stimulated with human IFN-γ at a concentration of 100 U/ml (Boehringer Mannheim, Mannheim, Germany) for 24/48 h and analyzed using immunocytochemistry, semiquantitative RT-PCR, and Western Blot. The following molecules were studied: lysosomal proteases (cathepsins B and L), cellular adhesion molecules (neural cell adhesion molecule [N-CAM]), apoptosis-related genes (Fas/Apo1, Bcl-x(l)), and cell cycle related-protein p21, all of which are known to be upregulated by IFN-γ. We also studied other proteins not known to be activated by IFN-γ, such as transcription factors involved in myogenesis (MyoD, myogenin), calcium-dependent proteases (calpain), and structural proteins (β-actin, desmin, and gelsolin).

**Immunocytochemistry**

Cells in culture were fixed in acetone before and after IFN-γ treatment and after incubation with hydrogen peroxide and a blocking solution containing 1% bovine serum albumin, 10% normal human serum, and 3% normal goat serum. The slides were further incubated with primary monoclonal antibodies (10 μg/ml) to calpain, cathepsins B and L, STAT1, desmin, and major histocompatibility complex (MHC-I) (Table 1). A secondary goat anti-mouse IgG antibody peroxidase conjugate was then applied (Jackson ImmunoResearch, West Grove, PA). The reaction was developed with diaminobenzidine (Vector Laboratories, Burlingame, CA).

**Western Blot Analysis**

Cytoplasmic extracts were prepared using a variant of the method by Schreiber et al (21). Human muscle cultures were scraped and washed 3 times in phosphate buffered saline (PBS) and resuspended in buffer A (10 mM N-2-hydroxyethylpiperazino-N-2-ethanesulfonic acid (HEPES), pH 7.9, 10 mM KCl, 0.1 mM ethylenediaminetetraacetic acid and 0.1 mM ethyleneglycoltetraacetic acid, and 1 mM dithiothreitol) containing the following protease inhibitors: 1 mM PMSF, 33 μg/ml apro- tinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A. After 10 min, an equal volume of buffer A containing 0.2% NP-40 was added and allowed to stand for 15 min and nuclei were spun down. The supernatant was collected, labeled as cytoplasmic extract, and stored at −80°C. The protein content of the cytoplasmic extract was determined by performing the Bio Rad Protein Assay (Bio Rad, Richmond, CA).
Equivalent quantities of proteins obtained, as described above, from nonstimulated cells or from cells stimulated with IFN-γ were subjected to electrophoresis on a 7% SDS-polyacrylamide gel (22) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA) (23). Blots were blocked for 2 h in 5% nonfat dry milk and incubated with the commercially available antibodies shown in Table 1 diluted in 3% nonfat dry milk in PBS for 1 h, washed 3 times with the same solution, and incubated with peroxidase conjugated goat anti-mouse antibody or goat anti-rabbit diluted 1/20,000 (Pierce, Rockford, IL) in 3% nonfat dry milk in PBS. After extensive washing, the blots were developed using enhanced chemiluminescent reagent according to the manufacturer’s instructions.

Semiquantitative RT-PCR

The expression of different messenger RNAs (mRNA) in human muscle culture (after treatment with IFN-γ) in nontreated cells and cultures stimulated with IFN-γ plus fludarabine was studied by means of semiquantitative RT-PCR. Total RNA was isolated from muscle cells in culture using a quick total RNA isolation kit (Pharmacia, Uppsala, Sweden) according to the manufacturer’s instructions. One microgram of RNA was reverse transcribed (1 h at 42°C, 5 min at 94°C) using oligo dT (Promega, Madison, WI), 75 mM KCl, 50 mM Tris-HCl, 3 mM MgCl₂, 10 mM dithiothreitol, 500 M each dNTP and 20 units of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, BRL, Bethesda, MD) in a final volume of 20 μl. The whole reverse transcription reaction was used as a template for PCR using the primers shown in Table 2. Each reaction also included 2.5 U/μl Taq DNA polymerase using buffer (BRL) and 500 M each of dNTP to a final volume of 100 μl. To facilitate the detection of changes in the levels of STAT1 mRNA after the different treatments, 30, 25, and 20 cycles (15 sec at 94°C, 15 s at 62°C, and 30 s at 72°C) of PCR were performed for each sample to check the exponential phase of the reactions. All PCR products were analyzed by 2% agarose gel electrophoresis with ethidium bromide staining.

Jurkat T cells as a Control for IFN-γ Production

Jurkat T cells (ATCC TIB-152) were grown in RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate, and supplemented with 10% fetal bovine serum. 4 × 10⁶ Jurkat cells/ml were incubated with 1 μg/ml phytohemagglutinin (PHA) (Sigma) and 50 ng/ml of phorbol myristate acetate (PMA) (Sigma). This double stimulation was necessary to promote the production of IFN-γ by Jurkat cells (24). Cells were harvested to perform RT-PCR of IFN-γ using the conditions mentioned above and the following primers: IFN1 5’GTTTCTTGGCTGTTACTG3’ and IFN2 5’GACGTTTCAGCCATCCTTG3’.

Inhibition of STAT1 Signaling Using Fludarabine

To further confirm that the effects of IFN-γ were transduced via STAT1, semiquantitative RT-PCR was performed in human muscle cultures in the conditions described above, but after previous treatment with the nucleoside analogue fludarabine (18). Briefly, human muscle cells cultures were grown to confluence

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**Table 2**

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<th>Primer/Interval</th>
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<th>Reverse primer</th>
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Fig. 1. Semiquantitative RT-PCR amplification products obtained from the RNA of human muscle culture before (lanes 1, 3, 5, 7, 9, 11, 13) and after 24 h of IFN-γ (2, 4, 6, 8, 10, 12, 14). The mRNA analyzed is indicated on the top. PCR was run for 20 cycles (exponential phase of the reaction). Primers for STAT1 were used in all samples as a control for upregulation after IFN-γ treatment (arrowheads).

Fig. 2. Loading control for RT-PCR. The RNA used in the semiquantitative PCR was checked using a third pair of primers for β-actin in the same reaction. The resulting bands show an increase of STAT-1 and cathepsin L after IFN-γ treatment, whereas β-actin levels remain unchanged.

until formation of myotubes was observed. Cultures were treated with fludarabine (Beneur, Schering, Germany) at 75 mg/m² for 24 h before treatment with IFN-γ. The cells were then left with fludarabine for a further 24/48 h after challenge with IFN-γ. Finally, the cultures were harvested and total RNA extracted for semiquantitative RT-PCR, Western blot, and immunocytochemistry.

RESULTS

Human Muscle Culture

mRNA and proteins were analyzed before and after treatment with IFN-γ at 24 and 48 h. These genes and protein products were grouped in the categories described below.

Genes Involved in Muscle Fiber Damage

After 24 h of IFN-γ stimulation, mRNA levels of cathepsins B and L increased (Fig. 1A). Because STAT1 was also increased, RT-PCR was performed, including a third pair of primers for β-actin, which is not activated by STAT1, as an internal control; mRNA levels of STAT1 remained unchanged as expected (Fig. 2). Western blot showed an increase in cathepsins B and L after 48 h but not after 24 h (Fig. 3A, B, respectively). Calpain mRNA (Fig. 1A) and protein levels remained unchanged after IFN-γ treatments.

Proteins Involved in Muscle Regeneration

Destructive changes in muscle are often accompanied by attempts at regeneration (20, 25–27). For this reason, we studied markers of regeneration and early myogenic differentiation. There was a very low upregulation of MyoD mRNA corroborated by Western blot after 48 h (not shown). N-CAM and AchR (Fig. 1B) mRNA levels did not change after 24 h of treatment with IFN-γ.

Structural Proteins

No upregulation of mRNA levels of either desmin, β-actin, or utrophin after 24 h or 48 h of IFN-γ stimulation were observed (Fig. 1A, B, respectively). These results were confirmed by Western blot analysis.

Proteins Involved in Apoptosis and Cell Cycle

Fas (CD95/APO-1) is known to transduce a signal to the nucleus in order to trigger apoptosis and is upregulated by IFN-γ in other cell systems (28, 29). However, our PCR and Western blot results showed no changes in Fas expression (Figs. 1A, 3C). Nevertheless, slight upregulation of Bcl-x(l) mRNA and protein (Figs. 1A, 3C) (30), which protects the cell from apoptosis, were observed.

P21 is a protein that stops the cell cycle by inhibiting cyclin dependent kinases. This protein is known to be upregulated after IFN-γ treatment via STAT1 (31). Semiquantitative PCR (Fig. 1B) showed p21 mRNA to be increased.
IFN-γ/STAT1 UPREGULATE CATHEPSINS IN HUMAN MUSCLE CULTURE

Fig. 3. Cytoplasmic extracts of human muscle culture were separated in a 10% polyacrylamide gel and transferred to PVDF membrane. Lanes 1 and 3 are controls. Lanes 2 and 4 are cells stimulated with IFN-γ. Loading control is shown at the bottom representing β-actin stained with Coomassie Blue in the gel after transfer. A: Upregulation of STAT1 (91 Kda) and cathepsin L (39 Kda) after 48 h of IFN-γ stimulation (lanes 2 and 4). B: Upregulation of STAT1 (91 Kda) but not of cathepsin L (39 Kda) after 24 h of IFN-γ stimulation (lanes 2 and 4). C: Increased expression of Bclx-l (26 Kda) and basal Fas levels (45 Kda) after 48 h of IFN-γ stimulation (lanes 2 and 4).

Controls for the Action of IFN-γ

IFN-γ induces MHC-I expression in cultured muscle cells (2, 32, 33). As expected, β2-microglobulin mRNA increased after 24 h IFN-γ stimulation (Fig. 1B). This result was confirmed by immunocytochemistry.

STAT3, another member of the family of signal transducers and activators of transcription, which is not activated by IFN-γ, was also studied. Results of RT-PCR showed no upregulation after 24 h stimulation (Fig.1B). The same results were obtained by Western blot after 48 h (data not shown). These results corroborate that the action of IFN-γ is also specific for STAT1 in muscle tissue.

Expression of IFN-γ by Muscle in Culture after Stimulation with External IFN-γ

The results of RT-PCR showed that human muscle cultures do not express autologous IFN-γ either constitutively or after being challenged with external IFN-γ. As a positive control for IFN-γ expression, Jurkat cells were used which, after being challenged with PHA and PMA, displayed a dramatic increase in IFN-γ mRNA expression (Fig. 4).

Immunohistochemistry on Human Muscle Culture

Immunocytochemistry on muscle cultures demonstrated increased staining for STAT1, cathepsins B and L, and MHC-I (33) after IFN-γ stimulation for 48 h, whereas calpain remained unchanged. The staining pattern was cytoplasmic and smooth in all cases except for cathepsin B, which displayed a granular pattern (Fig. 5). The immunohistochemical results fully correlated with RT-PCR and Western blot results.

Fig. 4. RT-PCR amplification products from unstimulated human muscle culture (lane 1), human muscle culture stimulated with IFN-γ (lane 2), unstimulated Jurkat T cells (lane 3), and Jurkat T cells stimulated with PHA + PMA (lane 4). Amplification of IFN-γ mRNA was only observed in Jurkat cells stimulated with PMA and PHA. β-actin was amplified in all samples.

Fludarabine-Induced Inhibition of STAT1 Signaling

Upregulation of mRNA for STAT1 and catL following IFN-γ stimulation was reduced by pretreating cells with fludarabine for 24 h before stimulation with IFN-γ (Fig. 6). A prolonged treatment (24 h after IFN-γ treatment) gave the same result. Fludarabine also blocked MHC-I
Fig. 5. Human muscle culture immunostained with a monoclonal antibody to cathepsin B before (left) and after 48 h of IFN-\(\gamma\) stimulation (right). Cathepsin B increased after IFN-\(\gamma\) stimulation, displaying a patchy pattern in the cytoplasm of myotubes (magnification \(\times\) 200).

expression on myotubes stimulated with IFN-\(\gamma\) (not shown).

Immunohistochemistry on Human Muscle Biopsies

The immunocytochemical studies on frozen sections from muscle biopsies of DM patients revealed that all atrophic perifascicular muscle fibers stained strongly for cathepsins B and L, STAT1 (Fig. 7A, B), and calpain (not shown). This confirms a previous study (17) that reported increased expression of cathepsins B and H as well as calpain and cathepsin L in DM. In contrast, biopsies from patients with polymyositis showed positive staining for STAT1 in the inflammatory infiltrates but these were negative for cathepsin L. None of the controls showed positive staining for either STAT1 or cathepsins B and L. MHC-I was upregulated in perifascicular fibers in DM (not shown).

DISCUSSION

This study examines the expression in skeletal muscle of several genes known to be regulated by IFN-\(\gamma\) in other cell systems. We studied human muscle culture and frozen sections from muscle biopsies of patients with IM and controls. We observed upregulation of the lysosomal proteases catB and catL, but not of the genes involved in apoptosis, regeneration, and structural proteins. Fludarabine reduced the IFN-\(\gamma\)-induced upregulation of STAT1 and catL. All of the results indicate that IFN-\(\gamma\)/STAT1
Fig. 7. Serial sections of fresh frozen muscle biopsy from a patient with DM stained with an antibody to STAT1 (A) and cathepsin L (B). Perifascicular fibers positive for STAT1 were also positive for cathepsin L. Arrowheads show 4 different areas of coexpression of both proteins (magnification 400).
plays a significant role in the upregulation of some proteolytic enzymes in vitro and that the expression of cathepsins in perifascicular muscle fibers observed in DM is an active process triggered by IFN-γ/STAT1.

The finding that STAT1 is highly expressed in atrophic perifascicular muscle fibers in DM and that IFN-γ is able to activate STAT1 in human muscle culture (13) opened a new field of investigation into this aspect of the immunopathology of DM.

We studied endogenous proteases as possible culprits of perifascicular atrophy. We found mRNA upregulation of cathepsins but not of calpain in our primary muscle cultures. Our immunocytochemical studies showed increased expression of cathepsins B and L and of calpain mainly in perifascicular atrophic fibers in DM but not in other IM, as previously reported (17). Our results in human muscle culture are in accord with those of other studies that report the upregulation of catB and L by IFN-γ in other cell systems (34). However, in our human muscle culture, calpain, which belongs to a family of extra-lysosomal Ca²⁺-dependent proteases, was not upregulated by IFN-γ, suggesting that other cytokines delivered to the medium or a phenomenon of low-grade ischemia may promote the expression of this protease.

We did not find upregulation of Fas antigen after IFN-γ treatment of human muscle cultures using RT-PCR and W-B. Increased expression of Fas after IFN-γ stimulation has been described by several authors in SV40-transformed human keratinocytes and CD34+ human marrow cells (28, 29). However, we did observe slight upregulation of Bcl-x(l) mRNA, which is a protector of apoptosis and is known to be upregulated by IFN-γ via STAT1 (30). Upregulation of Bcl-x(l) has been found in inflammatory cells in IM (35) and it has been shown that in DM, the few Fas-expressing muscle fibers and inflammatory cells are protected by the anti-apoptotic protein Bcl-2, which belongs to the same family as Bcl-x(l) (36).

In the same line of results it has been reported that both muscle fibers and inflammatory infiltrates in IM biopsies express Fas-associated death domain-like IL-1-converting enzyme-inhibitory protein (FLIP), an anti-apoptotic molecule (37). Furthermore, the absence of apoptotic features both in the inflammatory infiltrates and the muscle fibers of patients with IM has been also reported (38). In summary, our in vitro findings and the above-mentioned studies seem to rule out an apoptotic process in the immunopathogenesis of IM.

MHC class I antigens are known to be constitutively expressed at low levels in cultured human myoblasts and myotubes and are upregulated after IFN-γ stimulation (2). We also detected increased expression of MHC-I in human myotubes in culture after 48 h of IFN-γ stimulation. We found basal expression of β₂-microglobulin and upregulation of its mRNA after 24 h of stimulation with IFN-γ, which was in agreement with the above-mentioned results. We also observed increased expression of MHC-I in the perifascicular muscle fibers in DM patients muscle biopsies, as previously reported (39). In DM, according to our results, STAT1 expression in perifascicular muscle fibers represents the triggering of MHC-I expression by the action of cytokines (mainly IFN-γ) released by inflammatory infiltrates.

Muscle cells have been reported to secrete cytokines such as IL-6 (40). This phenomenon of autocrine production by nonimmune cells has also been described in sensory neurons from fetal rat dorsal root ganglion (41). However, in our system, external IFN-γ was unable to activate IFN-γ production by muscle cell culture. This suggests that the IFN-γ, which affects muscle fibers, is probably produced by perimysial inflammatory infiltrates present in the muscle, and not as an autocrine production by muscle. Another possible explanation for the expression of STAT1 in muscle, especially when perifascicular infiltrates are not detected, may be that IFN-γ production can take place at sites far from the fascicle periphery as part of systemic dysfunction, and enter the muscle via the bloodstream. Also, a sustained activation of STAT1 after IFN-γ degradation could explain the absence of infiltrates in some cases. It has previously been shown that STAT1 is not activated in ischemic processes (13). Our results suggest that the perifascicular atrophy observed in the muscle biopsies of patients with DM is multifactorial and includes IFN-γ, among other factors such as ischemia or calpain activation.

Experiments performed using fludarabine, a STAT1 blocker, further confirmed that IFN-γ stimulation was transduced via STAT1. Fludarabine interferes with STAT1 phosphorylation, preventing not only its translocation to the cell nucleus but also the activation of transcription of its target genes, STAT1 being one of them. This observation could have therapeutic implications since a partial blockade of proteases such as catL in the perifascicular muscle fibers in resistant DM might benefit these patients.

In summary, this study further indicates that the activation of lysosomal proteases (cathepsins B and L) occurs via IFN-γ/STAT1 in human muscle and is a factor that contributes to secondary damage of the perifascicular muscle fibers in patients with DM.

REFERENCES


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