Muscle Wasting Induced by HTLV-1 Tax-1 Protein

An in Vitro and in Vivo Study

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Besides tropical spastic paraparesis/human T-cell leukemia virus type-1 (HTLV-1)-associated myelopathy, the human retrovirus HTLV-1 causes inflammatory disorders such as myositis. Although the pathogenesis of HTLV-1-associated myositis is primarily unknown, a direct effect of cytokines or viral proteins in myocytotoxicity is suspected. We have developed an *in vitro* cell culture model to study the interactions between primary human muscle cells and HTLV-1 chronically infected cells. When HTLV-1-infected cell lines were added to differentiated muscle cultures, cytopathic changes such as fiber shrinking were observed as early as 1 day after contact. This was accompanied by alterations in desmin and vimentin organization, occurring in the absence of muscle cell infection but with Tax-1 present in myotubes. Cytopathic changes were also observed when infected culture supernatants were added to the muscle cells. Fiber atrophy and cytoskeletal disorganization were confirmed in muscle biopsies from two HTLV-1-infected patients with myositis. Transduction of cultured muscle cells with a lentiviral vector containing the HTLV-1 *Tax* gene reproduced such effects *in vitro*. The present data indicate that the myocytotoxicity that is observed in HTLV-1-associated myopathies can be due to a direct effect of the *Tax*-1 protein expressed in infected inflammatory cells, in the absence of muscle cell infection. (*Am J Pathol* 2005, 167:1609–1619)

by acquired muscle weakness and inflammatory infiltrates of muscle tissue.¹ According to clinical and histopathological features, they are divided into dermatomyositis, polymyositis, and sporadic inclusion-body myositis. Although autoimmune responses are believed to be operative in at least dermatomyositis and some polymyositis, target antigens have not been identified; however, for each disease group, T-cell products such as interleukin (IL)-1 (α and β), IL-6, and tumor necrosis factor (TNF)- α have been reported to be involved in the pathogenic process.² In humans and nonhuman primates, some myositis diseases have been associated, naturally or experimentally, with retroviral infection by human immunodeficiency virus, simian immunodeficiency virus, or human T-cell leukemia virus type 1 (HTLV-1).³ HTLV-1 is already known as the etiological agent of adult T-cell leukemia⁴ and of a neurological syndrome called tropical spastic paraparesis/HTLV-1-associated myelopathy (TSP/HAM).⁵ Numerous cases of myositis have been reported in HTLV-1-endemic areas including Japan, Caribbean Islands, and South America. Epidemiological, clinical, and histopathological observations suggest a possible involvement of HTLV-1 as an etiological agent of myositis. Epidemiological data are based on a report stating that in a group of 38 adult Jamaican patients with polymyositis, 63% were HTLV-1 seropositive.⁶ Similarly, 7 of 8 patients with polymyositis were reported to be HTLV-1-positive,⁷ as were 7 of 14 patients with dermatomyositis/polymyositis in French West Indies, as compared to 2.2% in the general population.⁸

Histopathological observations indicated that HTLV-1 infection can be associated with muscular inflammation characterized by direct invasion of the affected muscle

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Myositis is an inflammatory myopathy that constitutes a heterogeneous group of muscle disorders characterized

by HTLV-1-infected mononuclear cells⁷ and variation in fiber size with evidence of regeneration. Interestingly, HTLV-1 gp46 could be found by immunocytochemistry in many of the invading mononuclear cells,⁷ and polymerase chain reaction demonstrated the presence of the HTLV-1 Tax gene within the muscle.⁹ However, there was no evidence of infection of the muscle fibers by HTLV-1 either in HTLV-1-associated polymyositis^{10–12} or in HTLV-1-associated inclusion body myositis.^{13,14} These data suggest that HTLV-1-associated myositis would not be due to direct, persistent infection of the muscle fiber by the virus, but to a process induced by the HTLV-1-infected mononuclear cells that infiltrate the muscle. Inflammatory cells in HTLV-1 myositis could release cytokines and/or the viral Tax transactivator, which could be taken up by muscle fibers and induce cytopathic modifications. In this context we developed an *in vitro* model to study the interactions between primary human muscle cultures and HTLV-1-infected cell lines, to determine whether infection of muscle fibers could occur and/or if soluble factors could exert cytopathic effect, and to investigate the role of the Tax protein in muscle cells. In particular cytoskeletal markers such as desmin, which are involved in muscle integrity, differentiation, and degeneration/regeneration,^{15–19} were analyzed. The present results show a direct cytopathic effect of HTLV-1-infected cell lines on muscle cells, accompanied by cytoskeletal disorganization. These results correlate with *in vivo* data that we obtained from muscle biopsies of HTLV-1-infected patients with myositis and demonstrate a role for Tax-1 protein in these phenomena.

Materials and Methods

Cells and Culture Conditions

Satellite cells (ie, myogenic precursor cells that persist in postnatal and adult muscle) were originally isolated from the quadriceps of a 5-day-old infant, as previously described (CHQ5 cells)²⁰ and in accordance with the French legislation on bioethics. The cells were cultivated in Ham's F-10 supplemented with 50 μ g/ml of gentamicin and 20% fetal calf serum and were trypsinized when they reached half confluency. Differentiation of satellite cells into myotubes was induced in Dulbecco's modified Eagle's medium supplemented with 50 μ g/ml of gentamicin, 100 μ g/ml transferrin, and 10 μ g/ml insulin.²⁰

For nonadherent cells, we used human HTLV-1-transformed T-cell lines C91/PL, MT2, and C81-66. The MT-2 cell line is derived from bone marrow CD4⁺ T lymphocytes of a healthy donor after co-cultivation with leukemia cells of an adult T-cell leukemia patient.²¹ The C91/PL²² and C81-66 cell lines²³ are derived from human umbilical cord blood T cells transformed by HTLV-1; both cell lines express the Tax-1 protein, but the C81-66 cell line does not produce viral particles, in contrast to C91/PL or MT2 cells, because it bears a defective proviral genome. CEM cells, a human HTLV-1-negative T-cell line derived from a patient with acute lymphoblastic leukemia,²⁴ were used as a negative control, as were the human T-cell leukemia

line Jurkat cells.²⁵ All nonadherent cells were grown in RPMI 1640 medium supplemented with 1 mmol/L glutamine, 10% heat-inactivated fetal calf serum, and antibiotics. Lymphocytes were adjusted to 10⁶ cells/ml 18 hours before the onset of each experiment. In preliminary experiments, Tax-1 protein could be detected in the supernatants from HTLV-1-infected cells (C91/PL, C81-66) by Western blotting after concentration (Amicon Millipore, Bedford, MA) and immunoprecipitation (M-280 Dynabeads; Dynal, Oslo, Norway) but not in the supernatant from CEM cells (data not shown).

Immunocytochemistry

Immunocytochemical analysis was performed by immunofluorescence using either a laser confocal microscope (LCSM 510; Zeiss, Jena, Germany) or a Leica fluorescence microscope (DMRB, Wetzlar, Germany). Muscle cells were grown on coverslips in 24-well plates, and once differentiated, HTLV-1-infected or noninfected cell lines were added (ratio, 1:1) for 1 or several days. In some experiments either supernatants from the different cell lines were added or HTLV-1 cell lines were co-cultured in a Transwell (Costar, Corning, NY) porous insert (0.4 μ m), as previously described.²⁶ For inhibition experiments, muscle cells were exposed for 15 minutes with serum from a TSP/HAM patient who was positive for HTLV-1 by Western blot. Control experiments were performed using a control serum shown to lack HTLV-1 antibodies by Western blot. C91/PL cell lines were then added to the muscle cell cultures, and co-cultures were kept for 1 or several days, before being processed for immunocytochemistry. Cultures were fixed in 4% paraformaldehyde for 20 minutes at room temperature. Staining was performed after incubation for 30 minutes with 10% normal goat serum diluted in phosphate-buffered saline (PBS), to avoid nonspecific antibody binding. Monoclonal antibodies directed against vimentin and desmin (DAKO, Glostrup, Denmark) were used as primary antibodies. Rabbit monoclonal antibody against cleaved caspase-3 (Asp175) was obtained from Cell Signaling Technology (Bedford, MA) and was used at a dilution 1:100. Antibodies to HTLV-1 proteins included: mouse monoclonal antibody to p24 (Cambridge Biotech, Worcester, MA), serum from an HTLV-1-infected TSP/HAM patient, or a monoclonal anti-Tax antibody (168A51-42; National Institutes of Health AIDS Research and Reference Reagent Program).

Specific secondary antibodies were either coupled with fluorescein or Texas Red (Vector Laboratories, Burlingame, CA) and were used according to the manufacturer's instructions. Antibodies were incubated in 0.05% saponin and 10% normal goat serum overnight at 4°C or for 1 hour at room temperature. After washes, preparations were mounted in Vectashield medium (Vector Laboratories) with or without 4,6-diamidino-2-phenylindole (DAPI) (to visualize nuclei). In some experiments, the actin cytoskeleton was visualized on fixed cell cultures using BODIPY-Phalloidin (Molecular Probes, Eugene, OR) according to the manufacturer's instructions.

Electron Microscopy

For scanning electron microscopy, muscle cells were grown on coverslips in 24-well plates and, once differentiated, co-cultured with MT2 or CEM cell lines. Twenty-four hours later cultures were washed in PBS and fixed in 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH = 7.2) overnight at 4°C. Cells were washed three times for 5 minutes (each time) in 0.2 mol/L cacodylate buffer (pH = 7.2), postfixed for 1 hour in 1% (w/v) osmium tetroxide in 0.2 mol/L cacodylate buffer (pH = 7.2), and then rinsed with distilled water. Cells were then dehydrated through a graded series of 25, 50, 75, and 95% ethanol solution for 5 minutes (each time). Samples were then dehydrated for 10 minutes (each time) in 100% ethanol followed by critical point drying with CO₂. Dried specimens were sputter coated twice with carbon using a Baltec Med010 evaporator. Preparations were then examined and photographed with a JEOL JSM 6700F field emission scanning electron microscope operating at 5 kV. To quantify cytopathic changes, muscle cell cultures were grown in the presence of MT2 or CEM cells or with supernatants of these cultures as described above. At day 1 after contact, cell cultures were processed for scanning electron microscopy. Fifteen randomly selected fields for each of these different cultures (more than 1000 cells) were processed with NIH Image J image analysis software, to assess the total area covered by the muscle cells.

Transduction with Lentiviral Vector

Transfer of HTLV-1 *Tax* gene into cultured muscle cells was performed using a flap lentiviral vector.²⁷ In this vector, a three-stranded DNA structure, the flap, is created at the center of the HIV-1 genome during reverse transcription and acts as a *cis*-determinant of HIV-1 DNA nuclear import transfer of HTLV-1 *Tax* gene. Briefly, muscle cells were preincubated with dextran polymer before adding 0.8 ng of the vector DNA (so-called Trip-Tax or Trip-Ø according to the presence or not of the *Tax* gene) to 1.5×10^5 cells in 200 µl of medium with slow agitation at 37°C. Six hours later 800 µl of medium were added per well and cells were further incubated for 24 hours before differentiation, as previously described.²⁷

Muscle Biopsies

Muscle biopsies were obtained during diagnostic or surgical procedures, in accordance with the French legislation on ethical rules. HTLV-1-infected patient 1 was a 40-year-old woman suffering from TSP/HAM who developed concomitantly a progressive weakness involving the upper and lower limb muscles, as previously reported.²⁸ HTLV-1-infected patient 2 was a 38-year-old French West Indian native woman who complained of asthenia, lower back pain, and sciatica and a progressive muscular weakness for 6 years that involved the lower proximal and upper limb muscles, as previously described.¹³

Figure 1. Effect of HTLV-1-infected cell lines on cultured primary human muscle cells (myotubes). Satellite cells were differentiated into myotubes, then medium alone (A), or HTLV-1-infected MT2 cells (B, C), CEM cells (D), or MT2 supernatant (E, F) were added. One day later, cultures were processed for scanning electron microscopy. Original magnifications, $\times 800$.

For both patients, HTLV-1 infection was confirmed by Western blot analysis showing the complete pattern of anti-HTLV-1 antibodies in the plasma and by amplification of HTLV-1 proviral sequences from peripheral blood mononuclear cells; the diagnosis of sporadic inclusion-body myositis was performed according to clinical and histological parameters and electromyography.^{13,28} Control muscle biopsies, in the form of surgical waste, were obtained from healthy patients during orthopedic surgery. Immunocytochemistry for desmin and vimentin was performed as described above on cryostat sections of frozen tissue, as previously described.²⁸

Results

In Vitro Experiments

Effect of HTLV-1-Infected Cell Lines on Muscle Cell Cultures

Cultured myotubes, resulting from the differentiation of satellite cells, were incubated with HTLV-1-infected cell lines (MT2) or control T cell lines (CEM), and processed 1 day later for scanning electron microscopy. Whereas control cultures exhibited the typical morphology of differentiated myotubes (Figure 1A), cultures incubated with MT2 cells exhibited a strong binding of MT2 cells on the

Figure 3. Cytopathic effect of HTLV-1-infected cell lines on cultured muscle cells (**A–F**), and inhibition of this effect (**G–I**). **A to I:** Desmin immunoreactivity at day 2 after contact in control muscle cell cultures (**A**), or cells in contact with C91/PL (**B**), C81-66 cells (**C**), or supernatant from C91/PL (**D**) or C81-66 (**E**), or in contact with C91/PL cells co-cultured on the filter from a Transwell plate (**F**). Addition of an anti-HTLV-1 serum from an infected patient was able to revert this cytopathic effect in muscle cells in contact with C91/PL (**G**) or C81-66 (**H**) cells, whereas no effect is seen with a control human serum from a HTLV-1-seronegative patient (**I**). Original magnifications, $\times 700$.

Table 1. Quantification of the Surface Area Covered by Muscle Cells in the Presence of HTLV-1-Infected Cell Lines (MT2) or Noninfected Cell Lines (CEM Cells) or Their Respective Supernatants

Control	+MT2 cells	+MT2 supernatant	+CEM cells	+CEM supernatant
15.62 10^6	6.31 10^6	5.13 10^6	13.37 10^6	13.44 10^6

Muscle cell cultures were grown in the presence of MT2 or CEM cells or supernatants of these cultures as described in Materials and Methods. At day 1 after contact, cell cultures were processed for scanning electron microscopy. Fifteen randomly selected fields for each of these different cultures were processed with NIH Image J image analysis software, to assess the total area covered by the muscle cells (arbitrary units).

differentiated myotubes and a dramatic shrinkage of these myotubes (Figure 1, B and C). In contrast, there was no binding of CEM cells observed or shrinkage of muscular fibers in the presence of these cells (Figure 1D). When cell cultures were incubated with supernatant of MT2 cells, a cytopathic effect was also observed (Figure 1, E and F). No effect was observed with supernatant of CEM cells (data not shown). We have quantified these cytopathic effects on scanning electron microscopy pictures from control muscle cell cultures and co-cultures with MT2 or CEM cells (or supernatant) (15 pictures/each

case). As shown in Table 1, an important decrease (more than 60%) in the surface area covered by muscle cells is observed in the co-cultures with HTLV-1-infected cells such as MT2 compared to control cultures as well as co-cultures with noninfected CEM cells. The supernatant from MT2 cells induced the same decrease, whereas the supernatant from CEM did not have significant effect (Table 1).

The cytopathic effect induced by HTLV-1-infected cell lines was also investigated by immunofluorescence of the cytoskeleton components, desmin and vimentin. One day

Figure 2. Effect of HTLV-1-infected cell lines on cultured muscle cells. Desmin immunofluorescence in cultured muscle cells in the absence (**A**) or presence (**B**) of MT2 cells (phase contrast picture is added in **B** to localize MT2 cells, day 1 after contact. **C–F:** Desmin immunofluorescence in muscle cells cultured in the presence of C91 cells at days 2 (**C**) and 8 (**D**) after contact or in the presence of CEM cells at day 8 after contact (**E**). **F, G:** Vimentin immunofluorescence in muscle cells cultured in the absence (**F**) or presence (**G**) of C91/PL cells at day 1 after contact. **H:** Visualization of p24 viral antigens (arrows) in muscle cells cultured in contact with C91/PL cells for 1 day. A phase contrast picture is added to visualize the contact between C91/PL and muscle cells. Original magnifications: $\times 1000$ (**A, B, H**); $\times 800$ (**C–G**).

after contact of MT2 cells with myotubes, a dramatic decrease in myotube diameter (Figure 2B) was observed as compared to control cultures (Figure 2A). This decrease was accompanied by an alteration in the pattern of desmin immunoreactivity, especially in the regions of close contact between MT2 and the muscle cells (Figure 2B). Fiber atrophy and alterations in desmin immunoreactivity could also be seen with C91/PL cells on the first day after contact and this was even increased at day 2 after contact (Figures 2C and 3B). These changes could be observed in three different experiments. At day 8, a severe atrophy of the myotubes was observed (Figure 2D). No cytopathic effects could be seen when the muscle cells were in contact with CEM or Jurkat cells even at day 8 after contact (Figure 2E). An increase in vimentin immunoreactivity was observed in myotubes in contact with MT2 cells at day 1 after contact (Figure 2G), in contrast to the very low level of immunoreactivity in control (Figure 2F) or to myotubes in contact with CEM cells (data not shown). No infection of the muscle cells was detected in the presence of MT2 or C91/PL cells as assessed by immunofluorescence for gag p24 viral antigens in the myotubes, while immunoreactivity could be easily detected in infected lymphoid cell lines (Figure 2H).

Assessment of apoptosis in muscle cells in contact with C91/PL cells was performed at day 3 by immunocytochemistry using an anti-cleaved caspase 3 antibody. On more than 270 muscle cells counted from 15 microscopic fields, no significant difference was observed in caspase 3 immunoreactivity between control muscle cell cultures (7.66% immunoreactive cells) and muscle cell cultures in contact with C91/PL (6.64% immunoreactive cells).

To study the possible effect of soluble factors and/or Tax protein, muscle cells were put in contact with C91/PL or C81-66 cell lines, or with supernatants from these cell lines, or co-cultured with C91/PL cells separated by a filter in Transwell plates. As shown in Figure 3, the C81-66 cell line is able to induce a cytopathic effect at day 2 after contact (Figure 3C) similar to that observed with the C91/PL cell line (Figure 3B) in comparison to control cells (Figure 3A). A cytopathic effect was also observed with the supernatant of C91/PL or C81-66 cell lines (Figure 3, D and E, respectively), or when C91/PL cells were grown on the filter of the Transwell plates (Figure 3F).

Inhibition of the Cytopathic Effect

In addition, the cytopathic effect of C91/PL and C81-66 cell lines could be reverted when anti-HTLV-1 antibodies from an HTLV-1-infected patient serum was added to the medium because no major changes in cell morphology and in desmin organization could be seen at day 3 after contact with C91/PL or C81-66 cells in these conditions (Figure 3, G and H, respectively). In contrast, control serum from an HTLV-1 Western blot-seronegative individual had no effect, whereas the cytopathic effect could be visualized with C91/PL cells (Figure 3I) as well as with C81-66 cells (data not shown).

Figure 4. Detection of Tax-1 protein in muscle cells by immunofluorescence, with a monoclonal mouse antibody. After 3 days of contact of myotubes with C91/PL (**A**, **B**) or C81-66 cell lines (**C**), Tax immunoreactivity was found in lymphoid cells and in atrophied muscle fibers (**arrows**). No signal is obtained in control experiment (**D**). Original magnifications, $\times 800$.

Presence and Effect of Tax-1 in Cultured Muscle Cells

The presence of the Tax-1 protein within the muscle cells in culture could be detected by immunocytochemistry at 2 days after contact with C91/PL (Figure 4, A and B) or C81-66 (Figure 4C), but with a weaker staining. No signal was seen in the control (Figure 4D) or when the first antibody was omitted (data not shown).

Figure 5. Effect of Tax expression within cultured muscle cells. Muscle cells were preincubated with dextran polymer before being transduced with a Trip-Tax lentiviral vector containing the Tax gene (**B, D**) or a Trip-Ø vector (**C**). **A–C:** Visualization of desmin (immunofluorescence) at day 4 after transduction. **D:** Visualization of actin cytoskeleton (phalloidin-Bodipy568-78 staining, red), Tax protein (immunofluorescence, green), and nucleus (DAPI, blue). Original magnifications, $\times 1000$.

Muscle cells were transduced with a Trip-Tax flap lentiviral vector, and at day 4 after transduction significant cytopathic modifications could be observed in transduced muscle cells (Figure 5B). At the same time, control or Trip-Ø-transduced muscular cells exhibited classical morphology and desmin immunoreactivity pattern (Figure 5, A and C, respectively). Cytopathic changes in Trip-Tax-transduced cells included cell rounding and shrinkage, as well as a decrease in desmin immunoreactivity (Figure 5B). Double staining with labeled phalloidin (to visualize actin cytoskeleton) and anti-Tax antibody (indirect immunofluorescence) revealed that degenerating cells exhibited Tax-1 immunoreactivity in the nucleus or cytoplasm (Figure 5D). No Tax-1 immunoreactivity was found when primary antibody was omitted or in control or Trip-Ø-transduced muscular cells (data not shown).

In Vivo Experiments

Immunocytochemical studies were then performed on muscle biopsies from HTLV-1-infected or noninfected patients. In muscle biopsies from a healthy individual, desmin immunoreactivity exhibited a typical diffuse punctate staining on transverse sections (Figure 6, A and C). In contrast, biopsy from one HTLV-1-infected patient exhibited fibers with reduced diameter and

dense and compact desmin staining (Figure 5B) in one case; in the second biopsy, areas of fibers with a normal diameter showed decreased and diffuse staining (Figure 6D). This corresponded to regions with important cellular infiltrates, as visualized by DAPI staining (see Figure 6F in comparison to the control, Figure 6E). Assessment of vimentin expression indicated that whereas only very weak staining was observed in control biopsies (Figure 6G), a dramatic increase was observed in some fibers from HTLV-1-infected patient biopsies, especially in areas with important cellular infiltrates (Figure 6, H and J).

Discussion

Some evidence has been suggested by epidemiological data about the etiological role of HTLV-1 in myositis,^{6–8,29} but there is no direct evidence of a causative effect of HTLV-1-infected cells on myositis. However, some reports describe histopathological changes in muscles from HTLV-1-infected patients with polymyositis^{12,30–32} or inclusion-body myositis,^{13,14,28} even when not associated with TSP/HAM.³³ In the present study, to see whether HTLV-1-infected cell lines could induce cytopathic changes on muscle

Figure 6. Visualization by immunofluorescence of desmin and vimentin expression in muscle biopsies from HTLV-1-infected and healthy control (HTLV-1-seronegative) patients. Muscle biopsies were obtained during diagnostic or surgical procedure, from HTLV-1-infected patients suffering from TSP/HAM and muscle weakness (patient 1) or muscle weakness (patient 2), or from HTLV-1-seronegative healthy patient. Sporadic inclusion-body myositis was diagnosed for both HTLV-1-infected patients.^{13,28} Frozen muscle biopsies were sectioned in a cryostat, fixed, and processed for indirect immunofluorescence for desmin or vimentin as described in Materials and Methods. **A, C:** Desmin, control; **B, D:** desmin, HTLV-1-infected, cases 1 and 2. **E, F:** DAPI staining of nuclei on biopsy sections adjacent to control (**E**) or HTLV-1-infected patient 2 (**F**). **G:** Vimentin, control; **H:** vimentin, HTLV-1-infected, case 1. **I, J:** DAPI staining of nuclei on biopsy sections adjacent to control (**I**) or HTLV-1-infected patient 1 (**J**). Original magnifications, $\times 600$.

cells, we took advantage of human primary muscle cell cultures that are able to differentiate into myotubes. We show that HTLV-1-infected cell lines are able to induce

cytopathic changes in differentiated cultures of these muscle cells, in the absence of infection. This absence of infection has already been reported *in vivo* for ob-

servations on muscle biopsies from HTLV-1-infected patients with myositis,^{10,11,14,28} except in one report concerning a patient also infected with HIV.³² A possible explanation for the absence of infection of muscle cells can be correlated to the fact that the ubiquitous glucose transporter Glut-1, which has been reported as a putative receptor for HTLV-1,³⁴ is not normally expressed in skeletal muscle.³⁵ To our knowledge, only one study has dealt with the *in vitro* interactions between HTLV-1-infected cells and human cultured muscle cells,¹² but according to different culture protocols. In this case, cultures of muscle cells from HTLV-1-infected patients with polymyositis were exposed to autologous peripheral blood lymphocytes, and no infection of muscle cells could be observed up to 3 weeks after contact. However, the percentage of HTLV-1-infected lymphocytes cultured with the muscle cells was very low and never more than 20%.

The cytopathic changes that were observed in this study could provide a basis for understanding the muscle wasting that is observed during HTLV-1-associated myositis, including atrophy and degeneration of muscle fibers^{7,9,13,36–39} at the sites of mononuclear inflammatory infiltrates. The fiber shrinkage that was observed in muscle cell cultures in the presence of HTLV-1-chronically infected cell lines in this study suggests that there is a direct effect of the infected cells, in the absence of muscle fiber infection. Because it has been suggested previously that after persistence of virus in tissues other than muscle, autoaggressive T cells could cause T-cell-mediated and MHC-I-restricted myocytotoxicity,^{12,28} our data provide an additional mechanism that could be involved in HTLV-1-associated myositis.

Present results indicate that morphological alterations could be seen as early as day 1 after contact, whereas no morphological changes were seen with noninfected T-cell lines such as CEM or Jurkat, even after 6 days of contact. Such *in vitro* changes were not observed in the previously cited study,¹² and this is not surprising because the experimental conditions were different between this study and ours. The muscle cell death that was observed in our study was not due to apoptosis, because no differences were observed in immunoreactivity for the 17-kd cleaved caspase-3, an effector caspase of the apoptotic pathway,^{40,41} between control muscle cell cultures and those in contact with C91/PL or C81-66 HTLV-1-infected cell lines. This is in agreement with the *in vivo* data on muscle biopsies from HTLV-1-infected patients with myositis, in which apoptosis was detected only in infiltrated lymphocytes but never in the muscle fibers.²⁸

In the present study fiber atrophy was accompanied by changes in the intermediate filament organization. This effect was specific because it was not observed with non-HTLV-1-transformed T-cell line such as Jurkat or CEM cells. This is of particular concern because desmin has been reported to be crucial for maintaining the architectural and functional integrity of skeletal muscle⁴² and is involved in several myopathies.⁴³ For example, the introduction of a null mutation into the desmin gene by homologous recombination in a mouse model induced

muscle weakness, variability in muscle fiber diameter, macrophage infiltration, and necrosis,⁴² thus providing a possible link with HTLV-1-associated myopathies. Interestingly, these features have also been described in the muscle biopsies from HTLV-1-infected patients with myositis.^{13,28,31} We also observed desmin disorganization *in vivo*, in muscle biopsies from HTLV-1-infected patients with myositis, and within foci of inflammatory infiltrates.

The fact that cytopathic changes were observed with the supernatant of HTLV-1-infected cell lines suggests the implication of soluble factors. The HTLV-1 Tax protein and/or cytokines could be candidates for such a role, especially if we consider that C91/PL and C81-66, which produce the same effects, have been reported to express Tax-1, IL-6, IL-1 α , and TNF- α .^{44,45} Interestingly, the C81-66 cell line has been described as an HTLV-1-nonproducing cell line. This suggests that the effect we observed would not be due to the virus itself or to muscle cell infection, but either to Tax-1 or to the cytokines produced by the infected or Tax-1-activated cell lines. In this respect, inhibition of the cytopathic effect obtained with an anti-HTLV-1 serum from a patient, even with an HTLV-1-infected cell line that only produces Tax-1 in the medium as a viral protein (C81-66), reinforces the major role played by Tax-1. It had been shown previously that Tax-1 was present in the culture media of HTLV-1-transformed cells and was able to increase the expression of some cytokines, such as TNF- α and - β , and cellular genes that contain nuclear factor (NF)- κ B regulatory sequences.^{46–49} Evidence for secretion of Tax-1 in the extracellular environment of transfected cultured cells has been reinforced by a recent study.⁵⁰ *In vivo*, Tax-1 has been reported to be present in the muscle of an HTLV-1-infected patient with polymyositis,³² and the Tax-1 sequence has been found by polymerase chain reaction in DNA extracted from lesions of patients with HTLV-1-associated polymyositis.⁵¹ It has also been reported that HTLV-1 Tax mRNA is produced in mononuclear cells infiltrating the muscle of an HTLV-1-infected patient with sporadic inclusion body myositis.¹³ Thus, the role of Tax-1 in the cytopathic changes that we observed *in vitro* could be related to the *in vivo* observations, providing for the first time direct evidence for a role of Tax-1 in HTLV-1-associated myositis. In respect to the results we obtained in desmin disorganization *in vitro* as well as *in vivo* and to the possible role of Tax-1 in this process, it should also be noted that in a transgenic mouse model that expressed Tax-1, this latter was found in oxidative muscle fibers that developed severe progressive atrophy.⁵² Moreover, Tax-1 was localized in structures resembling degenerating Z bands, where desmin is located to maintain muscle cell integrity.¹⁷ In addition, the fact that we could reproduce these cytopathic features including desmin modifications by transduction of muscle cells with a Tax lentiviral vector, which has been shown to keep both pathways of Tax transactivation (CREB and NF- κ B) functional,²⁷ provides a strong argument for the role played by Tax-1 in HTLV-1-associated myositis. Tax-1 could exert its effect through a general myocytopathic

effect, for example through a mechanism of induction of cytotoxic factors such as TNF- α ,⁴⁹ as suggested by the up-regulation of proinflammatory cytokines in cultured microglial cells transduced by Tax-1;⁵³ another possibility for Tax-1 action would be through direct interaction with desmin, as shown with the neuronal intermediate filament protein α -internexin,⁵⁴ although up until now no direct interaction has been described between Tax-1 and desmin.⁵⁵

We further looked for vimentin expression in muscle cell cultures in the presence of HTLV-1-infected cell lines. Vimentin has been chosen both as a marker of degenerative and regenerative changes in muscle,⁵⁶ with a developmental expression pattern inversely correlated to desmin,⁵⁷ but also because Tax-1 is already known to activate the human vimentin gene.⁵⁸ We found that in the presence of HTLV-1-infected cell lines, vimentin expression was increased in muscle cells compared to controls. This can be correlated to the *in vivo* increase in vimentin expression described in muscle fibers of HTLV-1-infected patients with myositis. These data are compatible with a role of Tax-1 observed in our *in vitro* model similar to that we had observed *in vivo*. Such an increase in vimentin expression in muscle biopsies, which is linked to cycles of necrosis and regeneration in injured muscles^{15,59} could provide new keys in HTLV-1-associated myositis.

In conclusion, our report provides a relevant *in vitro* model to study the pathogenesis of HTLV-1-associated myositis and demonstrates a direct cytopathic effect of HTLV-1-infected cells associated with cytoskeletal reorganization, in the absence of muscle cell infection. Moreover, our data indicate that the viral protein Tax-1 is able per se to induce these cytopathic changes and may justify some therapeutic approaches against HTLV-1-associated myositis that specifically target Tax-1.

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