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Differential expression of the IL-1 system components during *in vitro* myogenesis: Implication of IL-1β in induction of myogenic cell apoptosis

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Abstract

We evaluated the expression of IL-1 system by normal human myogenic cells during *in vitro* myogenesis and the effect of exogenous IL-1β. Expression of IL-1α and β, IL-1 receptor antagonist (IL-1Ra), IL-1RI and II, IL-1R accessory protein (IL-1RAcP) and IL-1β converting enzyme (ICE) was studied by immunocytochemistry, immunoblotting, ELISA and RT–PCR. Cell proliferation was evaluated by [^{3}H]thymidine incorporation, cell fusion by flow cytometry and cell death by *in situ* end-labelling. Human normal myogenic cells constitutively produced IL-1β and ICE, with a maximum expression at time of cell fusion. IL-1Rs and IL-1RAcP expression reached a peak at time of commitment to fusion. Myogenic cells produced small amounts of IL-1Ra at latest stages of culture, and only the intracellular isoform. Exposure of cultures to exogenous IL-1β (1–5 ng/ml) induced myogenic cell apoptosis, without effect on cell proliferation or fusion. IL-1β-induced cell death was associated with morphological changes including spreading appearance of cells and alteration of cell alignment. We conclude that (1) human myogenic cells constitutively produce IL-1β; (2) IL-1 system components are differentially expressed during *in vitro* myogenesis; (3) IL-1 system participates to the coordinated regulation of cell density during normal myogenesis, which could serve to control the muscle mass *in vivo*.

Keywords: apoptosis; cell fusion; differentiation; interleukin-1; myogenic cell; myogenesis; skeletal muscle

Abbreviations: IL-1, interleukin-1

Introduction

Proinflammatory cytokines are produced by immunocompetent cells and by a large variety of other cells and tissues.[1,2] In addition to their role in the inflammatory reaction, they are likely implicated in a variety of physiological processes.[1–3] There is increasing evidence that skeletal muscle cells may express interleukins,[4–6] tumour necrosis factor (TNF-α)[7] and interferon-γ,[8] but the significance of cytokine production by muscle cells is largely unknown.

The interleukin-1 (IL-1) system includes (1) two biologically active 17 kD proteins, IL-1α and IL-1β, synthesized as 31 kD precursors called proIL-1α and β;[3] proIL-1β is cleaved by interleukin-1β-converting enzyme (ICE), an intracellular cysteine protease recently termed caspase-1;[9] (2) a natural competitive inhibitor, the IL-1 receptor antagonist (IL-1Ra), produced under two distinct isoforms, the secreted isoform (sIL-1Ra) and the intracellular isoform (icIL-1Ra), this latter lacking the signal peptide present in sIL-1Ra;[3] (3) two membrane-bound receptors, IL-1RI that transduces an intracellular signal and IL-1RII that does not.[10] Upon IL-1 binding, IL-1RI transduces intracellular signal by recruiting a 66 kDa membrane-bound protein, termed IL-1R accessory protein (IL-1RAcP), into a functional trimeric complex.[11,12] IL-1RII could act as a sink for IL-1,[10] or by forming a nonfunctional trimeric complex with IL-1 and IL-1RAcP, subtracting the coreceptor molecule from the signaling IL-1RI.[13]

In a series of previous papers, we assessed *in vivo* IL-1 expression by human muscle cells in normal and various pathological states using immunocytochemistry, immunoblotting, mRNA *in situ* hybridization, and reverse transcriptase-nested-polymerase chain reaction (RT–N–PCR).[6,14–16] IL-1 expression was mainly observed in the post-synaptic domain of motor-endplates in normal muscle,[15] and in myofibres showing myofibrillar breakdown in diseased muscles of patients with zidovudine myopathy, dermatomyositis, neurogenic target fibres and fibre atrophy.[6,14–16] This finding was consistent with the role ascribed to IL-1 in muscle proteolysis.[17–25] Another interesting finding was the frequent coexpression of IL-1 and NCAM in individual muscle fibres, suggesting that IL-1 could be associated with muscle cell regeneration[15,16] as previously suggested for IL-6 and LIF.[4]

To substantiate this hypothesis, we evaluated expression of the different IL-1 system components by muscle cells during the *in vitro* myogenic process. We also examined the effect of exogenous IL-1β on primary human muscle cell cultures.

Results

We studied IL-1 system expression by human myogenic cells during the myogenic differentiation process, using a

primary culture model previously described by our group.[26] In this model, myogenic cells proliferate from day 4, then align (day 8) and fuse into myotubes at days 12–16.[26] We first evaluated the expression of different IL-1 system components, including IL-1α, IL-1β, IL-1Ra, IL-1RI and II, IL-1RAcP, and ICE, using immunocytochemistry, immunoblotting, ELISA and RT–PCR, at different stages of the differentiation process. We observed a differential expression of IL-1 system components during the differentiation process, suggesting that IL-1 may play a role in myogenic cell differentiation. In the second part of our work, human myogenic cells were subjected to IL-1β alone, IL-1Ra alone and both IL-1Ra. We observed that IL-1β induces a decrease of cell density by inducing apoptosis, but without significant effect on cell proliferation and fusion. Our results suggest that IL-1 system participates to the coordinated regulation of cell density during normal myogenesis.

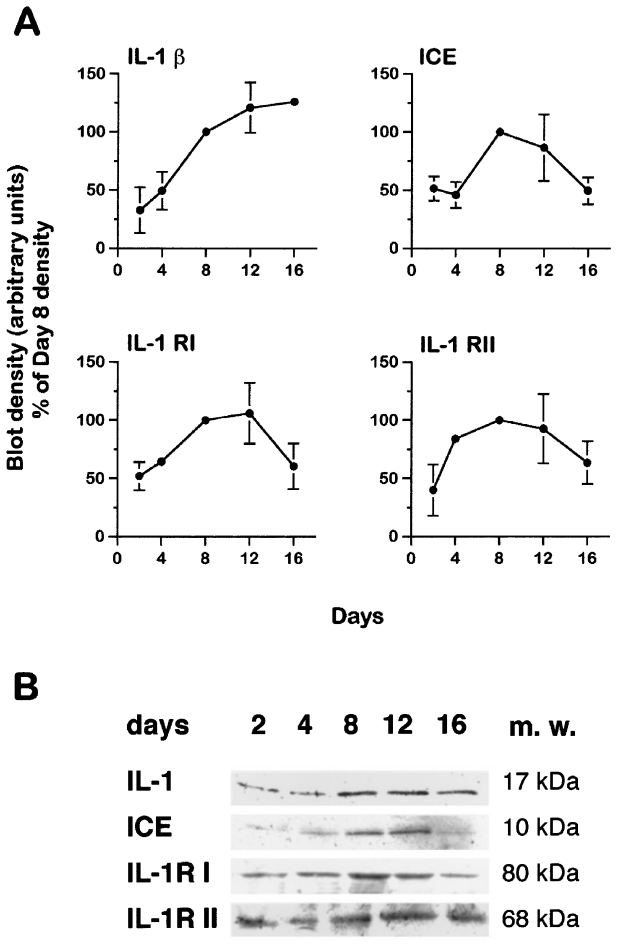

Figure 1 IL-1 system expression by human myogenic cells: immunoblotting. Cell extracts from day 2 to 16 of cultured human myogenic cells were proceeded for Western-blotting for IL-1β, ICE, IL-1-RI and IL-1-RII. (**A**) Densitometric analysis of the signal obtained by Western-blot of three different cultures. Results are expressed as percentages of the densitometric value obtained at day 8 of culture and are means $\pm$ S.D. (**B**) Example of the signal obtained for one culture

Production of IL-1α and IL-1β by human myogenic cells

IL-1β expression was detected by immunoblotting (Figure 1) from day 2 and by immunocytochemistry (Table 1, Figure 2B,F,H) from day 4 of culture. IL-1β mRNA was detected by RT–N–PCR from day 8 of culture (not shown). Immunoreactivity for IL-1β was observed in almost all cells, mainly around nuclei (Figure 2B,F,H). Maximal labelling was observed at days 8 and 12 of culture, i.e. at time of commitment of myogenic cells to fusion (Table 1, Figure 1). Since primary cultures may content few non-myogenic cells as fibroblasts,[26] double labelling procedure allows to assess IL-1β expression by desmin-positive cells. Immunofluorescence showed mononucleated cells positve for both IL-1β and desmin, i.e. myogenic cells (Figure 3), that confirmed the ability of myogenic cells to express IL-1β. Moreover, at later stages of culture, multinucleated cells, i.e. myotubes, expressed IL-1β (Figure 2H). The immunoreactive protein corresponded to the mature (17 kD) form of IL-1β, as assessed by immunoblotting (Figure 1).

ELISA confirmed the presence of IL-1β in cell extracts (Figure 4A). In the supernatant, ELISA revealed low amounts of IL-1β (up to 3.9 pg/mL) (Figure 4A). By comparison, IL-1β levels in supernatants of PBMC from healthy donors incubated with LPS (10 ng/ml) for 24 h were 60-fold those of myogenic cells when expressed as pg/mL/10^5 cells. No correlation was found between IL-1β levels in supernatants and the rate of cell mortality (Figure 4B). These data indicated that myogenic cells actively secreted low amounts of IL-1β.

Immunocytochemical expression of IL-1α was detected from day 4 of culture (Table 1, Figure 2C), but protein expression could not be confirmed by immunoblotting and ELISA. RT–N–PCR assessed the presence of IL-1α mRNA, at lower rates than IL-1β mRNA (not shown).

Cell expression and release of IL-1Ra by human myogenic cells

IL-1Ra protein was detected by immunocytochemistry (Table 1) and immunoblotting, only at late stage of culture (day 16). IL-1Ra was not detected by ELISA in culture supernatants. Accordingly, IL-1Ra RT–PCR showed that the mRNA of the intracellular isoform but not that of the secreted one, was expressed (Figure 5).

Table 1 Immunocytochemistry on human myoblasts

Days	2	4	8	12	16
IL-1α	–	+	+	++	+
IL-1β	–	+	+	++	++
IL-1-Ra	–	–	–	–	$\pm$
ICE	+	+	+	++	+
IL-1-RI	–	+	+	$\pm$	$\pm$
IL-1-RII	$\pm$	+	+	+	$\pm$

–: no staining; $\pm$: weak staining; +: strong staining; ++: very strong staining

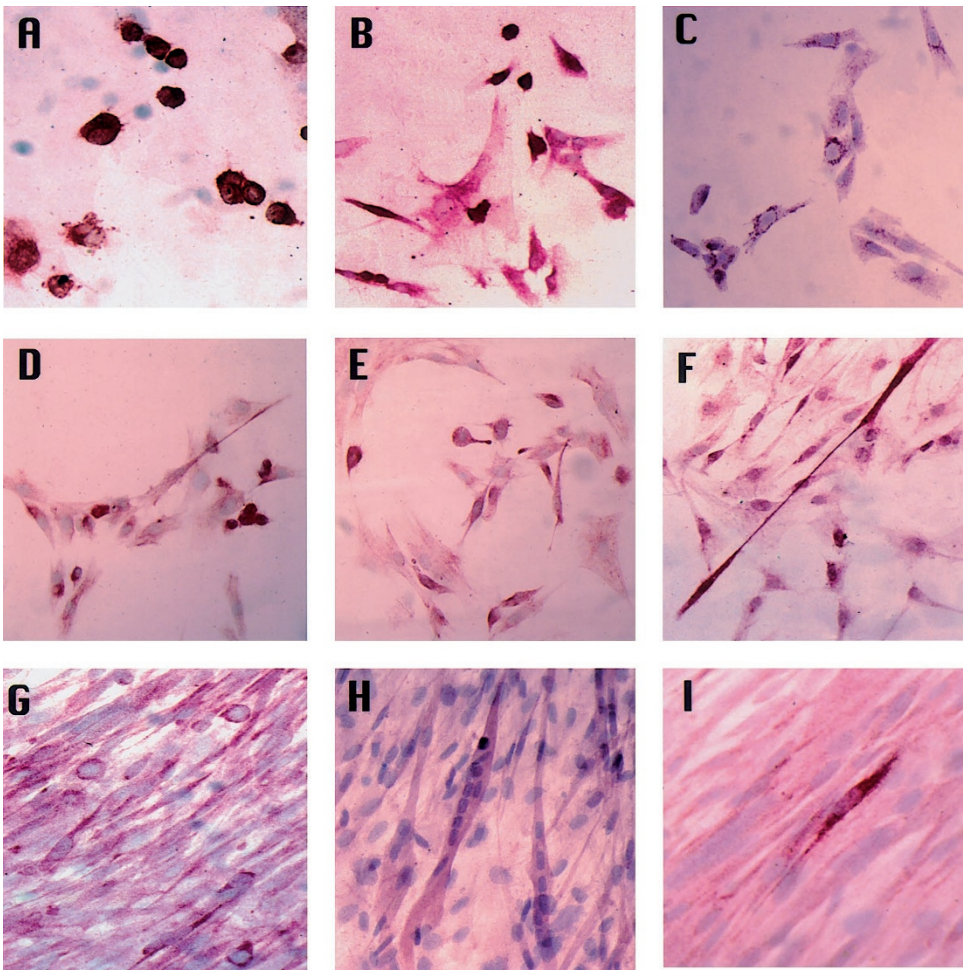

Figure 2 IL-1 system expression by human myogenic cells: immunocytochemistry. Cultured human myogenic cells were proceeded for immunocytochemistry for IL-1β, IL-1α ICE, IL-1-RI and IL-1-RII, revealed using APAAP. Expression of ICE at day 2 (**A**). Expression of IL-1β at day 4 (**B**), day 8 (**F**) and day 16 (**H**). Expression of IL-1α at day 4 (**C**). Expression of IL-1-RI at day 4 (**D**) and day 16 (**G**). Expression of IL-1-RII at day 4 (**E**) and day 16 (**I**). Magnification: $\times 400$

Expression of ICE by human myogenic cells

ICE was detected from day 2 of culture, i.e. before IL-1β, by both immunocytochemistry (Figure 2A, Table 1) and immunoblotting (Figure 1). The labelling strongly increased until day 8 and then decreased until day 16. ICE labelling was granular. Densitometric analysis of immunoblots indicated that at day 16, the level of ICE reached that detected at day 2 (Figure 1). ICE was expressed in its active form as assessed by: (1) immunoblots showing a band at 10 kDa, that corresponds to the p10 subunit of the enzymatically active heterodimer of ICE; (2) immunoblots showing the mature form of IL-1β predominantly; (3) the kinetics of immunocytochemical expression of ICE showing that ICE is expressed before IL-1β.

Expression of IL-1 receptors and IL-1RAcP by human myogenic cells

Weak immunocytochemical expression of IL-1RI was observed from day 2 of culture (Table 1). At day 4, almost all cells were still weakly labelled (Figure 2D). The expression strongly increased at days 8 and 12 and decreased at day 16 (Figure 1, Table 1). Until day 16, the most strongly labelled cells were small mononucleated myogenic cells (Figure 2G). IL-1RII was also detected by immunocytochemistry from day 2 (Table 1). IL-1RII expression plateaued from day 4 (Figure 2E) to day 12 of culture and then decreased (Table 1, Figure 1). At day 16, only a few mononucleated cells remained strongly labelled (Figure 2I). The pattern of immunolabelling of both type I and II IL-1Rs was diffuse. Immunoblotting showed that myogenic cells expressed IL-1RAcP and that the expression paralleled that of IL-1 receptors (Figure 6).

Effect of exogenous IL-1β and IL-1Ra on cultured human myogenic cells

Exposure to IL-1β alone significantly decreased the cell density in a dose-dependent way ($P<0.05$) (Figure 7A). Conversely, exposure to IL-1Ra alone tended to increase the cell density, but we failed to find a statistical significance for both concentrations (Figure 7B). When both IL-1β and

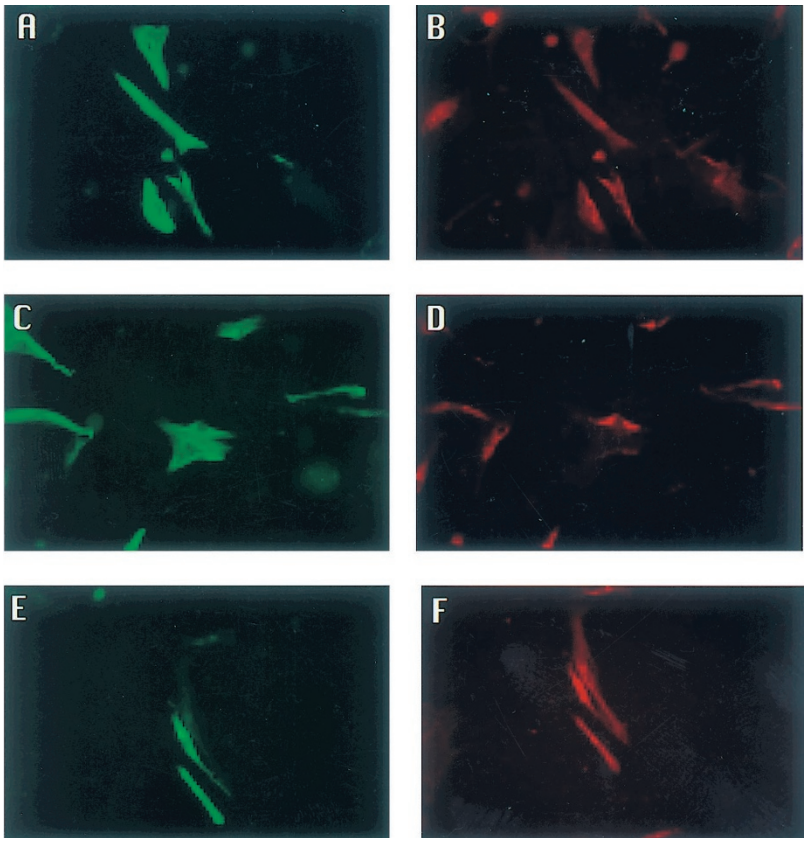

Figure 3 Desmin/IL-1β double labelling of myogenic cells: immunofluorescence. Three different clusters (**A**–**B**, **C**–**D**, **D**–**E**) of cultured human myogenic cells (day 4) were proceeded for immunofluorescence for desmin (FITC: **A**, **C** and **E**) and IL-1β (rhodamine: **B**, **D**, **F**)

IL-1Ra were added to cultures, cell density remained similar to controls (Figure 7C). The myotubes rate were not affected by exposure to IL-1β or IL-1Ra, whatever the concentration used (not shown). Proliferation assay did not show any difference in the incorporation of [^{3}H]thymidine between controls and treated cells (in per cent of control: IL-1β: 97.7$\pm$26.3; IL-1Ra: 89.4$\pm$19.4; IL-1β+IL-1Ra: 107.3$\pm$14.5). MGG staining revealed inconstant morphological changes after exposure to IL-1β, including spreading phenotype of myogenic cells and alteration of myogenic cell alignment. Morphological changes were not observed when cultures were subjected to IL-1Ra alone or to both IL-1β and IL-1Ra. As assessed by *in situ* end-labelling (ISEL) method, exposure to IL-1β induced a marked increase of the number of nuclei showing DNA breaks (Figure 8A,B).

Discussion

In the present study, we observed that (1) IL-1 is produced by unstimulated human myogenic cells *in vitro*; (2) the IL-1 system components are differentially expressed during the myogenic process; and (3) that significant cellular effects result from exposure to recombinant IL-1β, that can be inhibited by blocking IL-1 receptors by IL-1Ra.

Immunocytochemistry, immunoblotting and RT-nested-PCR have been previously used to assess *in vivo* expression or IL-1α and IL-1β by human muscle tissue.[6,14–16] None of these procedures could assess the exact source of IL-1 in muscle. Indeed, many cell sources other than muscle fibres may produce IL-1 in muscle tissue, including endothelial cells, vascular smooth muscle cells, fibroblasts and macrophages,[3] and external proteins, such as immunoglobulins, may enter and accumulate in diseased muscle fibres.[27] The present data establish that human myogenic cells can produce IL-1.

IL-1β was detected before IL-1β mRNA. On one hand, ICE was expressed from day 2 of culture, suggesting that early production of IL-1β may result from the cleavage of preexisting proIL-1β by ICE. In the other hand, IL-1β is known to stabilize and to increase the half-life of its own mRNA,[28] suggesting that IL-1β mRNA could be more easily detected when IL-1β is produced in a sufficient amount to stabilize it. IL-1β was found in culture supernatants, indicating that IL-1β acts in a paracrine fashion. IL-1RI expression peaked, and IL-1β expression stabilized, at days 8–12 when myogenic cells fuse into myotubes, suggesting that expression of the IL-1 system could be associated with the myogenic process. This view was supported by the peak of IL-1RAcP expression at day 12, IL-1RAcP expression being mandatory for IL-1/IL-1RI signalling. Consistently, exogenous IL-1β induced a

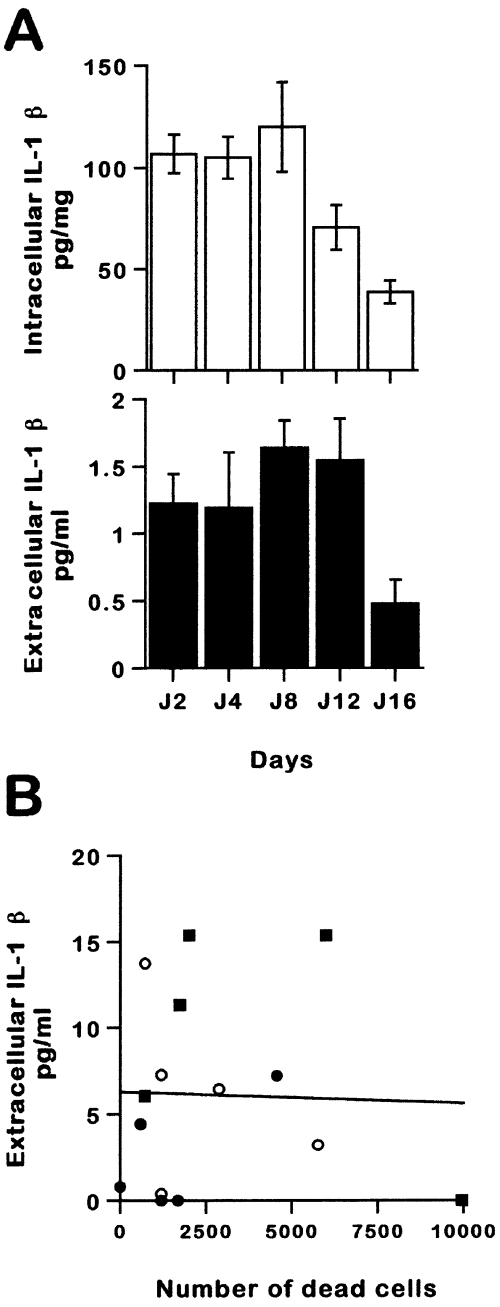

Figure 4 IL-1β secretion by human myogenic cells. (**A**) White columns: IL-1β concentration in human myogenic cell extracts. Black columns: IL-1β concentration in 24 h-culture medium of human myogenic cells. Results are means $\pm$ S.D. of three ELISA experiments run in triplicate. (**B**) Correlation between IL-1β concentration in 24 h-culture medium of human myogenic cells and the number of dead cells present in the culture estimated by Trypan blue dye exclusion. Each symbol represents one culture

decrease of myogenic cell density, associated with changes to a spreading cell phenotype and disorganization of the myogenic cell layer, all effects being prevented by addition of IL-1Ra.

In contrast to endothelial cells and fibroblasts,[29] myogenic cells produced intracellular IL-1Ra but not the secreted isoform, a finding previously shown for various epithelial cell types and human vascular smooth muscle cells.[30] Production of the intracellular form of IL-1Ra is suggestive of an autocrine role for IL-1Ra in myogenic cells, as a self-regulatory molecule that may antagonize a potent autocrine effect of proIL-1α.[30]

Exogenous IL-1α has been previously shown to decrease terminal differentiation of a myogenically determined cell line.[31] The effect of exogenous IL-1β on proliferation of cultured cells may differ according to the cell type.[32–41] To clarify the cellular effects of IL-1β on primary myogenic cell cultures, we used a [^{3}H]thymidine incorporation assay to assess whether IL-1 truly inhibits myogenic cell proliferation. This study showed that despite their significant effect on cell density, neither IL-1β nor IL-1Ra induced significant changes in myogenic cell proliferation. This was in keeping with the lack of IL-1 effect on the expression of p21,[42] the cyclin-dependent kinase inhibitor that induces irreversible cycle cell withdrawal of myogenic cells prior to fusion, as previously reported.[43] It appeared likely that apoptosis could account for the effect of IL-1 on myogenic cell density, since IL-1β has been previously shown to promote apoptosis through iNOS induction in other cell types,[3] and since NO can induce apoptosis of cultured myogenic cells.[44] Consistently, apoptosis was documented by *in situ* end-labelling and nuclear morphology, at days 4 and 8 of normal myogenic cell culture, and increased after exposure to IL-1β. It seems, therefore, likely that expression of the IL-1 system participates to the coordinated regulation of cell density during normal myogenesis, which could serve to control the muscle mass *in vivo*.[45]

Expression of IL-1 in myotubes in the absence of detectable apoptosis, and the striking morphological changes observed in cultures after exposure to IL-1β, indicated that IL-1β effects on myogenic cells were not restricted to limitation of cell density. ICE cleaves actin,[46] and IL-1 induces disorganization of the actin cytoskeleton of fibroblasts *in vitro*.[47] The spreading phenotype of myogenic cells exposed to IL-1β also reflected cytoskeletal changes. Such an effect of IL-1β is reminiscent of *in vivo* IL-1β expression by muscle fibres in pathologic states characterized by marked myofibrillar loss.[14–16] It is compatible with the induction by IL-1 of the ATP-ubiquitin-dependent proteolytic pathway[48] involved in contractile protein breakdown.[49]

In addition to its proteolytic effects, IL-1 is able to upregulate expression of several cell–cell adhesion molecules in various cell types,[3] and this could have participated to IL-1-induced clustering of myogenic cells. It has been shown that IL-1 added to myogenic cell cultures induces ICAM-I expression by myoblasts and myotubes.[50] This has been regarded as important to stick inflammatory cells.[50] It could also favour phagocytosis of apoptotic bodies and formation of neuromuscular junctions. Interestingly, IL-1 was previously detected in the postsynaptic domain of normal neuromuscular junctions,[15] suggesting that it could participate to initiation and maintain of synaptogenesis in the muscular system, as previously suggested in central nervous system.[51–53]

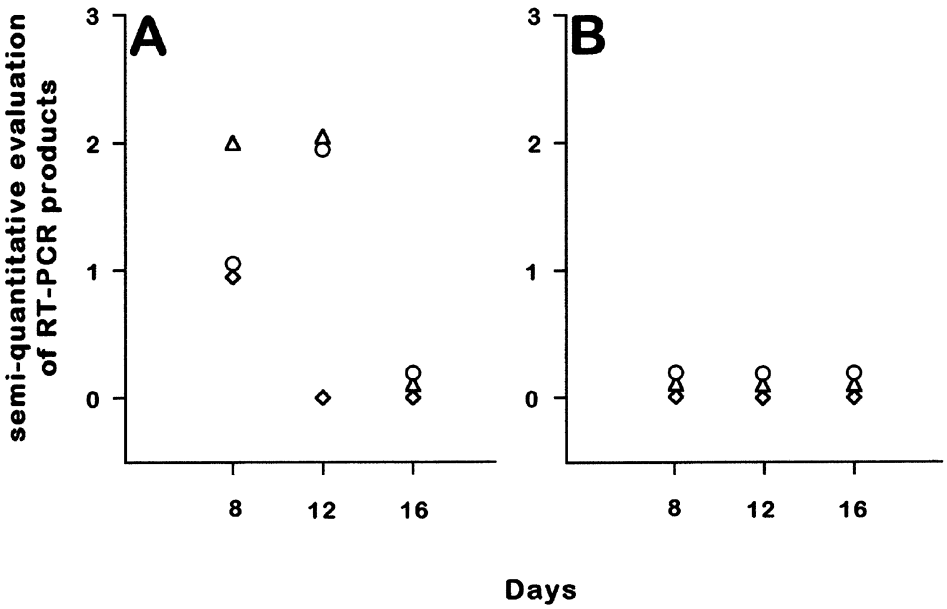

Figure 5 IL-1Ra RT–PCR. (**A**) Positive detection of IL-1Ra intracellular isoform mRNAs at days 8 and 12 of culture. (**B**) Lack of IL-1Ra secreted isoform mRNAs. Experiment performed on three different cultures. Quantification: Band intensity was graded as absent (undetectable)=0; feint=1+ (better visualized on UV light than on picture); moderate=2+; strong=3+

Materials and Methods

Cell culture

Human myogenic cells were obtained at time of orthopaedic surgery, in agreement with the French legislation, as previously described.[26] Briefly, muscle samples were mechanically and enzymatically digested and the satellite cells were cultured in HAMF12–15% foetal calf serum (FCS, Gibco BRL Life Technologies, Paisley, UK) with antibiotics (penicillin/streptomycin). The medium was routinely changed every 4 days. All the cells used in the experiments were passage 2 cells seeded at 2000 cells/cm^2. Three main stages can be observed in these conditions: proliferation (days 4–6), alignment (days 6–8) and fusion into myotubes (days 8–12).[26] Cells were evaluated at days 2, 4, 8, 12 and 16 of culture.

Cell density and fusion index

The cell density was determined by counting the total number of cells within the dish after treatment by trypsin at each date. The rate of cell death was evaluated by Trypan blue dye exclusion. To quantify the morphological differentiation, we evaluated both the rate of myotubes and the number of fusional events. The myotubes rate (MtR) in culture was defined: MtR=(number of myotubes)/(total number of myogenic cells). The number of fusional events was evaluated by measurement of the fusion index (FI). FI was defined according to Falzoni *et al*.:[54] fusion index (FI)=(number of nuclei within myotubes)/(total number of nuclei counted) $\times$ 100. To avoid any confusion in counting between binucleated myotubes and telophasic dividing cells, we considered as myotubes only cells with 3 nuclei or more. In case of cells grown in adherent conditions, the morphological evaluation of FI using MGG staining has proven cumbersome and at many times inaccurate due to major cell overlap at late stages of culture. In order to palliate these liabilities, MtR and FI were evaluated using flow cytometric analysis, carried out on a Coulter® EPICS® XL Flow Cytometry System and analyzed with XL System II® Software (Coulter Corporation, Miami, FL, USA). Since our study was performed on primary cultures of myogenic cells obtained from healthy donors, the cultured myogenic cells were diploid and therefore the cell content of DNA was proportional to the number of nuclei. Cell DNA content was evaluated by permeabilization followed by propidium iodide (PI) staining, using DNA-Prep® (Coulter) kit. For each sample, three parameters were obtained: forward scatter (FSC), indicating cell volume; side scatter (SSC), indicating cell structure; FL1 indicating the fluorescence channel detecting PI fluorescence (590 nm). FL1 showed a very reproducible pattern of several discrete fluorescence peaks, distributed regularly along the FL1 axis on the histogram (Figure 5), allowing an easy separation and precise quantitative rapid assessment of myogenic cell subsets, according to the fluorescence intensity of PI, and therefore the number of nuclei contained in each cell. Quantification of different subsets and calculation of FI was performed on about 10 000 cells for each sample. All experiments were carried out on at least four different cultures.

Immunocytochemistry

Expression of the different components of the human(h) IL-1 system was evaluated using polyclonal rabbit anti-hIL-1α, polyclonal rabbit anti-hIL-1β, polyclonal rabbit anti-hIL-1Ra (Genzyme, Cambridge, MA, USA), polyclonal rabbit anti-hIL-1RI (Santa Cruz Biotechnologies, Santa-Cruz, CA, USA), mouse monoclonal anti-hIL-1RII (Genzyme) and polyclonal rabbit anti-hICE p10 subunit (Santa Cruz). All antibodies, were diluted in Tris buffer saline (TBS) containing 0.1% bovine serum albumin (BSA). Cells grown on glass coverslips were rinsed with phosphate buffered saline (PBS), fixed in acetone for 10 min at room temperature, dried and incubated in TBS-3% BSA pH 7.6 for 1 h to block non-specific binding. Incubation with the primary antibodies was performed at 4°C overnight using the following dilution: anti-hIL-1α 1 : 25, anti-hIL-1β 1 : 25, anti-hIL-1Ra 1 : 100, anti-hIL-1RI

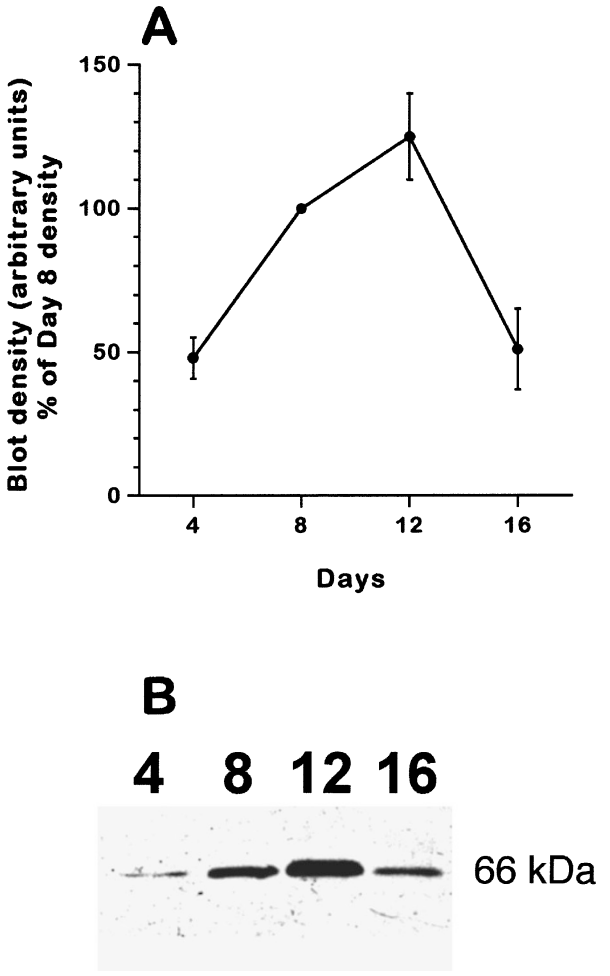

Figure 6 IL-1RAcP expression by myogenic cells. Cells extracts from days 4 to 16 of cultured human myogenic cells were proceeded for immunoblotting for IL-1-RAcP. (**A**) Densitometric analysis of the signal obtained by Western-blot of three different cultures. Results are expressed as percentages of the densitometric value obtained at day 8 of culture and are means ± S.D. (**B**) Example of the signal obtained for one culture

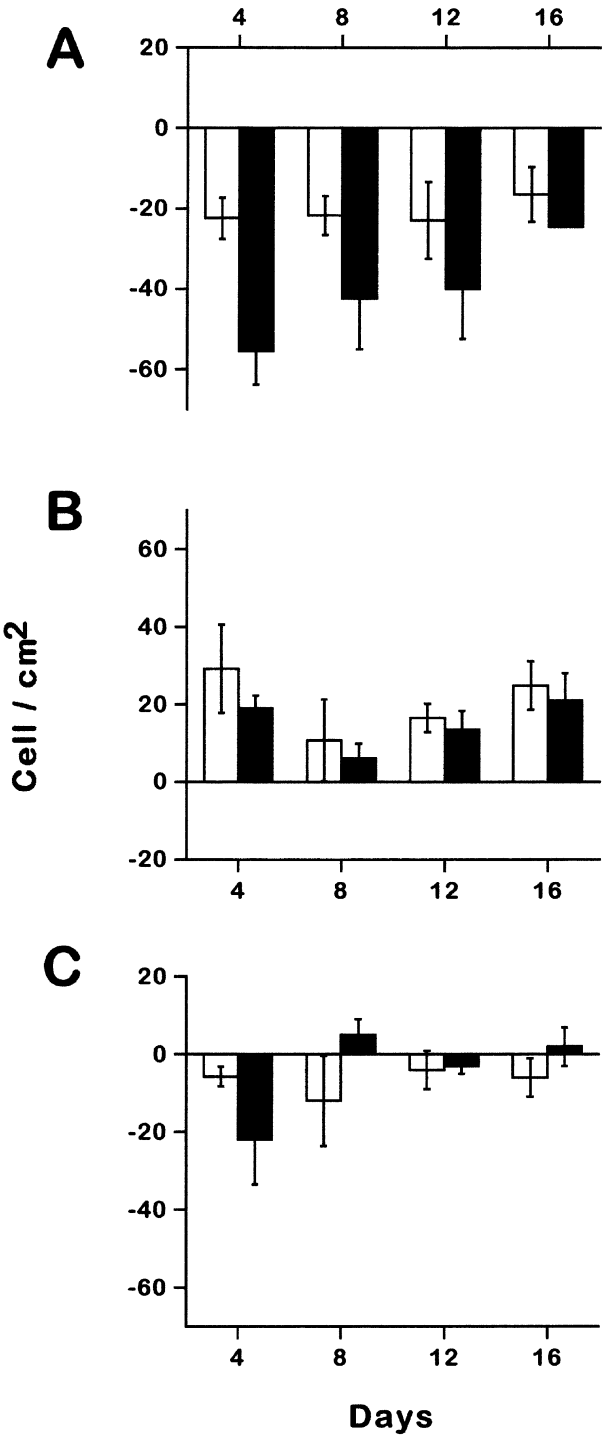

Figure 7 Effect of IL-1β and IL-1-Ra on human myogenic cell density. Human myogenic cells were treated either with IL-1β (**A**), IL-1Ra (**B**) or both (**C**) and were proceeded for the estimation of cell density. White columns: IL-1β: 1 ng/mL, IL-1Ra: 100 ng/mL; black columns: IL-1β: 5 ng/mL, IL-1Ra: 500 ng/mL. Results are expressed as a percentage of the number of cells in non-treated cultures and are means ± S.D. of four experiments run in duplicate

1 : 100, anti-hIL-1RII 1 : 100, anti-hICEp10 1 : 100. Further steps included: (1) link-step with rabbit anti-mouse immunoglobulins (Ig) (1 : 50, 30 min) in case of primary monoclonal antibodies, or with successively mouse anti-rabbit Ig (1 : 50, 30 min) and rabbit anti-mouse Ig (1 : 50, 30 min) in case of primary polyclonal antibodies; (2) incubation with alkaline phosphatase-conjugated mouse anti-alkaline phosphatase antibodies (APAAP 1 : 50, 30 min); and (3) visualization of the reaction products using Fast Red® (Sigma, St Louis, MO, USA). Coverslips were mounted on slides after counterstaining for 10 s with Harris haematoxylin. To assess the specificity of the immunostaining, the procedure was performed without primary antibody. Immunocytochemical experiments were run on primary cultures from five different subjects. In addition, detection of a coexpression of IL-1β and desmin was ensured by using a double labelling procedure and visualization by immunofluorescence (IF). Primary antibodies were polyclonal anti-hIL-1β (Genzyme; 1 : 25 in TBS-0.1% BSA, 30 min RT) and monoclonal anti-desmin (Novocastra; 1 : 25 in TBS-0.1% BSA, 30 min RT) antibodies. Detection of IL-1β labelling was ensured by rhodamine-conjugated pig anti-rabbit IgG antibody (Dako, Glostrup, DK; 1 : 20 in TBS-0.1% BSA, 30 min RT) and detection of desmin labelling by FITC-conjugated rabbit anti-mouse IgG antibody (Dako; 1 : 20 in TBS-0.1% BSA, 30 min RT).

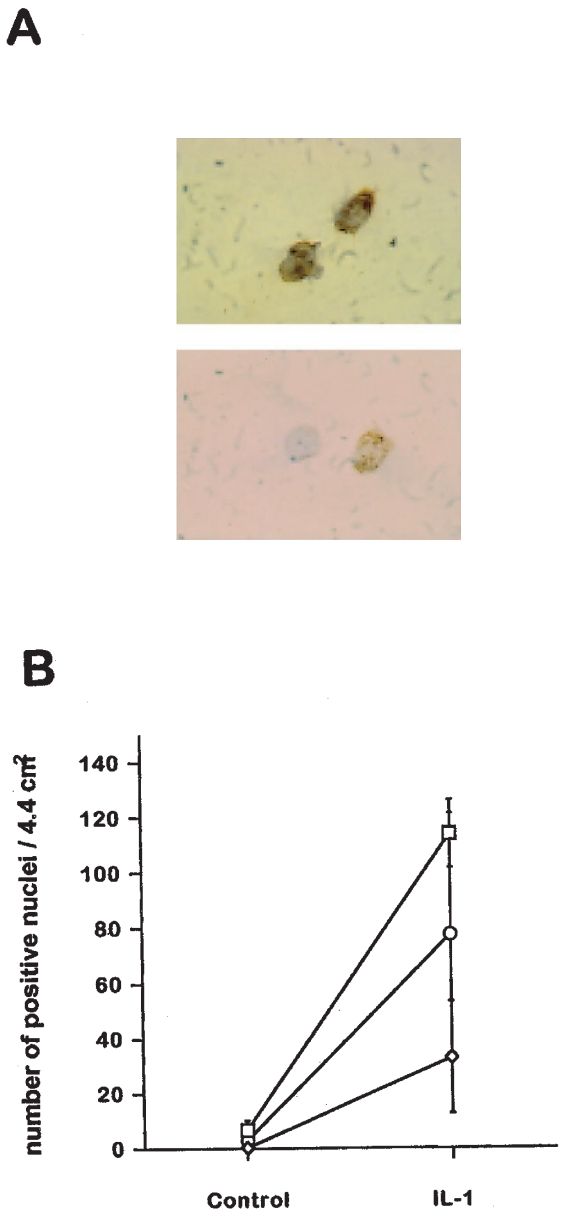

Figure 8 DNA breaks detection by *in situ* end-labelling (ISEL): effect of IL-1β on human myogenic cells. (**A**) Exposure to IL-1β: myogenic cells showing ISEL-positive nuclei (brown staining; no counterstaining) at day 8 of culture. Up: two positive nuclei; down: one positive nucleus, one negative. Magnification: $\times$400. (**B**) Quantification of DNA breaks detected by ISEL in three cultures after exposure to IL-1β. Quantification was carried out by counting the number of positive nuclei in a 4.4 cm^2-field (day 4: squares; day 8: circles; day 12: diamonds)

DNA breaks detection by *in situ* end-labelling

The *in situ* end-labelling (ISEL) method was performed using the ApopTag® Kit (Oncor, Gaithersburg, MD, USA) according to the manufacturer's recommendations, and a modified alkaline phosphatase assay, as previously described.[55] Briefly, cells grown on glass coverslips were washed with phosphate buffer saline, then processed with proteinase K. All samples were washed and treated with Equilibration Buffer and incubated sequentially with the working strength of TdT Enzyme for 1 h and the Stop/wash Buffer for 30 min. Sections were incubated for 30 min with peroxidase-labelled anti-digoxigenin antibody 1/100 (Boehringer, Mannheim, Germany) and reactivity was detected using diaminobenzidine (DAB).

Immunoblotting

Cells grown on 75 cm^2 flasks were rinsed with PBS, scraped and centrifuged at 250 $\times$ *g* at 4°C. Cell pellet was incubated with lysis buffer (CHAPS 1% w/v, NaCl 150 mM, TRIS 50 mM; EDTA 5 mM; phenylmethylsulfonyl fluoride (PMSF) 1 mM, pH 7.8) and gently shaken for 10 min on ice. The homogenate was centrifuged at 4000 $\times$ *g* for 10 min at 4°C, and the supernatant was kept at −80°C. Protein concentration was determined using the BCA kit from Pierce (Rockford, IL, USA). Aliquots corresponding to 20 μg of proteins were subjected to SDS–PAGE, followed by transfer onto nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). All antibodies were diluted in PBS-Tween 20 0.1% (PBST). Staining of the blotted membranes was performed as follows: (1) blockade of the non-specific binding by incubation with PBST-dry non-fat milk 5% for 2 h at RT; (2) overnight incubation at 4°C with the primary antibody; (3) link-step either with a biotinylated-mouse anti-rabbit Ig (1 : 1000, 1 h) in case of polyclonal primary antibody, or with a biotinylated-rabbit anti-mouse Ig (1 : 1000, 1 h) in case of monoclonal primary antibody; (4) incubation with streptavidin-biotin-peroxidase complex 1 : 500 1 h (Amersham, Buckinghamshire, UK). Revelation was performed using the ECL kit (Amersham). Detection of IL-1α, IL-1β, IL-1Ra, IL-1RI, IL-1RII and ICE was ensured by using the same antibodies as for immunocytochemistry, diluted 1 : 100. Detection of IL-1RAcP was ensured by using a mouse monoclonal anti-IL-1RAcP IgG1 (Transduction Laboratories, Lexington, KY, USA), diluted 1 : 1000. Controls ensured the lack of endogenous binding of the secondary antibody and the absence of peroxidase. Experiments were run at least on three different cultures for each point. Densitometric quantification of blots was performed using the NIH Image® 1.60 software and was expressed in percentages of arbitrary units.

RT–PCR

Detection of IL-1α and IL-1β mRNA was performed using a previously described RT-nested-PCR (RT–N–PCR) procedure.[6] For the present study we also design a RT–N–PCR procedure for detection of IL-1α mRNA. Outer primers for IL-1α RT–N–PCR have been previously published.[56] The inner primers were designed overlapping two consecutive exons of IL-1α to avoid any amplification of contaminating DNA. Sequences of inner primers were 5′-ACTCAGAGGAAGAAATCATCAAGC-3′ and 5′-CTCAGGCATCTCCTTCAGCAGCAC-3′. The probe used for IL-1α RT–N–PCR amplicons was 5′-AATGACGCCCTCAATCAAAG-3′. Detection of IL-1Ra mRNA was performed using a RT–PCR procedure, previously described by Beasley *et al*.[30] which allows to separate the two alternatively spliced isoforms, icIL1Ra and sIL-1Ra. Amplification of cDNA from myogenic cells was compared with that from human peripheral blood mononuclear cells (PBMC) from healthy donors incubated with IL-1β (10 ng/mL) for 24 h, which express both icIL1Ra and sIL-1Ra.[30] Amplification with primers specific for icIL-Ra and for sIL-1Ra yielded PCR products of respectively 510 and 533 bp.[30] PCR products were visualized under ultraviolet (UV) light on a 2% agarose gel stained with ethidium bromide (5 μg/mL), and were photographed using a Polaroid system (MP4, Osi, France). Band intensity was graded as

absent (undetectable)=−; 1+=feint (better visualized on UV light than on picture); 2+=moderate; 3+=strong.[6]

ELISA

Cells were cultured in 35 mm diameter Petri dishes. The medium was changed 24 h before cell treatment. The culture medium was removed and kept at −80°C. Protein extracts of cells were obtained as described above, in the Western-blot section and kept at −80°C. Protein concentration was determined using the BCA kit from Pierce. The concentration of IL-1α, IL-1β and IL-1Ra in the culture medium and in the cell fraction was determined using commercial ELISA kits (Immunotech, Marseilles, France). Detection threshold were 5 pg/mL for IL-1α and IL-1β and 15 pg/mL for IL-1Ra. In experiments run on three different cultures, IL-1α, IL-1β and IL-1Ra concentration was measured in supernatants using commercial kit (Immunotech) and in unconditioned culture medium. Concentrations were compared to the rate of cell death, evaluated by Trypan blue dye exclusion. To compare the level of released IL-1β to that obtained with cells assigned to IL-1β secretion, we measured the concentration obtained in the supernatant of PBMC from healthy donors incubated with LPS (10 ng/ml) for 24 h.

Incubation of cultures with IL-1β and IL-1Ra

Recombinant human IL-1β and IL-1Ra were purchased from PeproTech EC (London, UK). Cells grown in 2 cm^2 dishes were treated with IL-1β and/or with IL-1Ra, following two different procedures: (1) IL-1β 1 ng/ml and IL-1Ra 100 ng/ml; (2) IL-1β 5 ng/ml and IL-1Ra 500 ng/ml. The IL-1Ra concentrations were 2.5×10^4 and 1.25×10^5 fold higher than the highest concentration of IL-1 found in the culture medium of the cells (see Results). Assuming that 100 molecules of IL-1Ra are needed to block the binding of either IL-1β or IL-1α on IL-1 receptors,[57] these conditions ensure a large excess of IL-1Ra in the culture dish, even in presence of soluble IL-1 receptors in the culture medium. IL-1β and IL-1Ra were added 1 day after seeding and the medium containing IL-1β or IL-1Ra or both was changed every 24 h. At days 4, 8, 12 and 16 of culture, some dishes were treated to measure the cell density using and mortality using Trypan blue exclusion test, others to evaluate the FI using flow cytometry, as described *supra*.

Proliferation assay

Four days cultured cells (proliferation stage) were incubated with IL-1β (1 ng/ml) or IL-1Ra (100 ng/ml) or both in culture medium containing 2 mCi/ml [^{3}H]thymidine. After a 18 h incubation time, cells were washed with PBS, lysed in 0.1% SDS and precipitated with 5% trichloroacetic acid (TCA) (final concentration) for 45 min on ice. The precipitate was recovered using a filter (Millipore, AFPA 02500), washed twice with TCA 5%, and finally counted in a β-counter. The radioactivity measured in treated cells is expressed as percentage of the radioactivity incorporated in non-treated cells. Controls were cells cultured with unconditioned medium.

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