

Influence of cyclic intravenous pamidronate on proinflammatory monocytic cytokine profiles and bone density in rheumatoid arthritis treated with low dose prednisolone and methotrexate

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Abstract

Objectives

The aim of this work was to evaluate in a randomised double-blind prospective study the effect of pamidronate on intracellular monocytic cytokine profiles (IL-1, IL-6, TNF- α) and bone density in rheumatoid arthritis patients.

Methods

Twenty rheumatoid arthritis patients were treated for one year with methotrexate and a low dose of prednisolone. Double blind randomisation was performed for either IV pamidronate (at 3-month intervals) or placebo. The effect of pamidronate was evaluated on intracellular cytokine profiles (IL-1, IL-6, TNF- α), disease activity and bone mass measurements. The human monocytic cell line THP-1 was used to evaluate in vitro apoptosis by pamidronate.

Results

Spontaneous production of interleukin-1 α by patient blood monocytes was lower in the pamidronate group and was associated with an increase in bone density of the spine after 12 months of therapy. In vitro a dose-related increase in pamidronate induced apoptosis was found in THP-1 cells.

Conclusions

This prospective double-blind randomised study demonstrated that pamidronate therapy resulted in an increase of bone density despite treatment with steroids. This rise is associated with a suppression of interleukin-1 α production in monocytes of patients treated with pamidronate. Our in vitro experiments suggest that this anti-inflammatory effect could be due to an increase in the apoptosis of monocytic cells.

Key words

Rheumatoid arthritis, bisphosphonates, pamidronate, inflammation, osteoporosis, cytokines, monocytes, apoptosis, necrosis.

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Introduction

Patients with rheumatoid arthritis run a high risk of developing both generalised and peri-articular osteoporosis. The pathogenesis of this bone loss is complex. Important etiological factors might be the reduced mobility due to functional impairment and the production of inflammatory mediators such as interleukin-1 (IL-1), tumour necrosis factor (TNF) and interleukin-6 (IL-6) (1-4). Suppression of inflammation and prevention of joint damage are the main objectives of therapy and the basis of prevention for rheumatoid arthritis associated osteoporosis.

Low dose methotrexate (7.5 - 15 mg/week) is generally considered to be an effective treatment for the inflammatory symptoms of rheumatoid arthritis, but its consequences on bone metabolism remain unclear. Oral glucocorticoids are widely used as an anti-inflammatory treatment for patients with rheumatoid arthritis, but their effect on joint destruction is uncertain and they are associated with a dose-related risk for generalised osteoporosis. On the other hand steroids increase the mobility of patients, possibly cancelling out some of the negative effects on bone (5).

Bisphosphonates are strong inhibitors of osteoclast-mediated bone resorption. They are useful in the prevention and treatment of corticosteroid-induced osteoporosis (6, 7). However, in rheumatoid arthritis their effects on inflammatory mediators and bone resorption are unclear and remain to be elucidated.

In this study rheumatoid arthritis patients with active disease were treated with methotrexate in association with low dose prednisolone. Double blind randomisation was performed for either cyclic intravenous pamidronate or placebo. The aim of the study was to investigate the effect of pamidronate on cytokine profiles, clinical parameters of disease activity, radiological damage scores, and bone mass measurements. In addition, the mechanism of the possible anti-inflammatory effect of pamidronate was studied in the human monocytic cell line THP-1, by measuring the induction of apoptosis.

Materials and methods

Patients

Twenty patients with active rheumatoid arthritis were prospectively selected for this study. All patients fulfilled the diagnostic criteria of the American College of Rheumatology for the classification of Rheumatoid Arthritis (8). Active disease was considered to be present if all of following criteria were met: morning stiffness lasting more than 20 minutes; the presence of at least 6 painful joints and 3 swollen joints; and an ESR of more than 28 mm/1st hr or C-reactive protein of at least 1 mg/dL. Patients previously treated with steroids, methotrexate, bisphosphonates, fluoride or anabolic steroids were excluded, as were those with impaired renal function (serum creatinin >1.7 mg/dL), thyroid or parathyroid dysfunction, diabetes, active treatment for epilepsy or tuberculosis and those with a history of alcohol abuse.

In all patients treatment was started with methotrexate (7.5-15 mg/w) and a low dose of corticosteroids (5 - 10 mg/d). Double blind randomisation was performed for treatment with an intravenous infusion of either placebo or pamidronate 60 mg in 1000 mL glucose 5% over 3 hours, starting one day before the beginning of steroid and methotrexate therapy and subsequently every 3 months. All patients received 1000 mg of calcium daily and 5 mg of folic acid weekly. Vitamin D supplementation was provided when the baseline vitamin D level was lower than 15 ng/mL.

Study parameters were evaluated at inclusion and after one year of treatment. Informed consent was obtained from each patient and the study protocol was approved by the local ethical committee.

Disease activity, bone remodelling, bone densitometry and radiological damage scores

The erythrocyte sedimentation rate (ESR), peripheral white blood cell count and differentiation, and C-reactive protein (CRP) were determined. The Ritchie articular index, number of swollen joints, Visual Analogue Scale

(VAS), global disease activity score (DAS) as assessed by both the physician and patient, and the Health Assessment Questionnaire (HAQ) were evaluated (9). Plasma osteocalcin was measured by an RIA (Biosource), according to the manufacturer's instructions. The fasting calcium/creatinin ratio was calculated for each patient. The bone mineral density (BMD) of the patient's lumbar spine (L2-L4), hip and metacarpals of the hand were determined by a dual energy X-ray absorptiometry (DEXA) method (Lunar, type DP XL absorptiometer, Wisconsin, USA). Radiographs of the hands were scored for joint narrowing and erosions according to Van der Heyde's modification of Sharp's method (10).

Intracellular cytokine analysis

Cytokine analysis was performed as previously described by Verbruggen *et al.* (11). Briefly, peripheral blood mononuclear cells (PBMCs) were separated by Nycoprep density gradient from 30 mL of EDTA peripheral venous blood. Cells were concentrated at 5×10^6 cells/mL in AIM-V medium. PBMCs were cultured for 8 hours in a 5% CO₂ humidified atmosphere at 37°C. To obtain maximal cytokine production, a part of the cells were stimulated with LPS (5 µg/mL) and monensin (1 M) in AIM-V.

Intracellular IL-1, IL-6 and TNF- α in monocytes were determined using a two-color flow cytometric technique (11). After stimulation, 1 mL phosphate buffered saline without calcium and magnesium (PBS) was added to the cell suspension. Cells were spun for 5 minutes at 1000 g, and were resuspended at 1×10^7 cells/mL in PBS. To identify monocytes, the cells were incubated with anti-CD14-fluorescein isothiocyanate (FITC) for 15 minutes at room temperature. Subsequently the cells were fixed for 30 minutes with 4% paraformaldehyde in PBS. Cell membranes were made permeable with a 0.3% saponin solution in PBS, followed by incubation with anti-cytokine antibodies against IL-1, IL-6 and TNF- α for 30 minutes at room temperature. A total of 2,500 events were measured on a FACScan flow cytome-

ter (Becton Dickinson Immunocytometry Systems) and analysed with WinMDI software (provided by J. Trotter through <http://facs.scripps.edu/software.html>). Analysis gates were set on CD14-positive cells according to FITC emission and side scatter. Within this gate, intracellular cytokine production was evaluated. Results were expressed as the mean fluorescence intensity. Measurements were standardised using a LinearFlow™ Orange (530/560) Flow Intensity Calibration Kit. Isotype-matched irrelevant antibodies were used as negative controls.

THP-1 cell culture

The human monocytic cell line THP-1 was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1 M Hepes buffer, 100 units/mL of penicilline, and 100 µg/mL streptomycin. To ensure exponential asynchronous growth, the cells were split every third day to obtain a density that did not exceed 5×10^6 cells/mL. The cells were incubated at 37°C in a 5% CO₂ humidified incubator for 48 hr in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 1 M Hepes buffer, 100 units/mL of penicillin, 100 µg/mL streptomycin and enriched with CaCl₂ (final concentration 4.5 mM). Incubation was carried out in the presence or absence of pamidronate at concentrations of 10 µM, 100 µM and 300 µM (12).

Viability of THP-1 cell line

Before and after incubation, the viability of the cells was measured with propidium iodide (50 µg/mL in PBS) in a FACScan flow cytometer and analysed with WinMDI software. The results were expressed as the percentage of the propidium iodide negative viable cells.

Apoptosis of THP-1 cells

Annexin-V/propidium iodide staining. THP-1 cells were suspended in 100 µL Hepes buffer (containing 140 mM NaCl, 2.5 mM CaCl₂) and stained with 5 µL annexin-V-FITC and 10 µL propidium iodide (50 µg/mL) for 15 min at room temperature in the dark. After incubation, 400 µL Hepes buffer was

added. Cells were analysed in a FACScan flow cytometer with WinMDI software. The results were expressed as a percentage of the annexin positive and propidium iodide negative apoptotic cells.

DNA staining with 7AAD. One million THP-1 cells were suspended in 300

µL PBS and fixed by slowly adding 1.5 mL 80% cold (-20°C) ethanol. The cells were fixed for 30 minutes at 4°C in the dark and then spun at 1000 g for 10 min. The pellet was resuspended in 1 mL PBS and incubated at 37°C for 30 min. After centrifugation, the cells were stained with 20 µg/mL 7-amino actinomycin (7-AAD) in PBS for 15 min at 4°C in the dark. They were measured in a FACScan flow cytometer and analysed using WinMDI software. The results were expressed as a percentage of the hypoploid cells.

Light microscopy. Before and after 48 hours of incubation, a cytospin of THP-1 cells was made. The cells were stained with May-Grünwald-Giemsa stain (Auto-Hemacolor, Merck). Two hundred cells were scored as apoptotic if clear nuclear condensation and/or fragmentation (apoptotic bodies) was present. The results were expressed as a percentage of apoptotic cells.

Reagents

The following is a list of the reagents used and their sources.

AIM-V medium (Life Technologies, Paisley, Scotland), Mouse anti-human CD14 fluorescein isothiocyanate (FITC) (Becton Dickinson Immunocytometry Systems, Erembodegem, Belgium), Rat anti-human IL-1 phycoerythrin conjugated (RPE), mouse anti-human IL-6 RPE and mouse anti-human TNF- α RPE (Biosource International, Camarillo, CA, USA), Rat IgG2a RPE and mouse IgG1 RPE (Serotec Ltd, Oxford, England), LinearFlow™ Orange (530/560) Flow Cytometry Intensity Calibration Kit (L-7307 Lot: 5864 (Molecular Probes, Eugene, USA), Lipopolysaccharide (LPS L-3755 Lot 84H4017), E. Coli Serotype 026:B6 (Sigma Chemical Co, St. Louis, USA), Monensin (Sigma), Nycoprep (d = 1.077 mg/mL, Nycopmed, Oslo, Norway), Phosphate Buf-

ferred Saline without calcium and magnesium (PBS) (Life Technologies). Penicillin/Streptomycin (Life Technologies). Saponin S-7900 Lot 91 H0325 (Sigma). Paraformaldehyde (Sigma). IL-1 (Genzyme, Cambridge, England), IL-6 and TNF- (ReproTech, Rocky Hill, New Jersey). RPMI 1640 (Life Technologies). DMEM (Dulbecco's Modified Eagle Medium, Life Technologies). Hepes (Life Technologies). Bovine Calf Serum (Hyclone, Utah, USA). CaCl₂ (Merck, Darmstadt, Germany). Propidiumiodide (Sigma). 7-amino actinomycin (Molecular probes). Annexin-V-FITC (Pharmin-gen, San Diego, Ca, USA). Auto-Hemacolor (Merck). Ethanol (Merck). Pamidronate (Aredia, kindly provided by Novartis, Brussels, Belgium).

Statistical analysis

The Wilcoxon rank test and Man Whitney-U test were used where appropriate. A p value less than 0.05 was considered significant.

Results

Patient characteristics and disease activity parameters

The two treatment groups were comparable with regard to age, gender, disease duration and cumulative dose of prednisolone and methotrexate (Table I). Differences in the initial disease activity scores were not statistically significant between the pamidronate group and the placebo group (Table II) except for the ESR. The latter was due to one outlier.

After 12 months of therapy, significant decreases in the ESR and CRP were observed in all patients ($p = 0.05$) (Table II). The disease activity in both treatment groups was significantly lower after 12 months therapy. Scores for Ritchie's articular index, the number of swollen joints, the Health Assessment Questionnaire (HAQ), the Visual Analogue Scale (VAS), and the global disease activity score (DAS) both by the physician and patient were significantly decreased (Table II).

Bone remodeling, bone densitometry and radiological damage scores

After 12 months of therapy, osteocalcin

Table I. Patient characteristics (median + range).

	Pamidronate group (n = 10)	Placebo group (n = 10)
Age	56 (35 - 77)	62 (41 - 77)
Female/male	6/4	8/2
Disease duration in months	15 (1.5 - 240)	20 (1.5 - 250)
1 year cumulative dose of prednisolone in mg	2,744 (2,255 - 2,856)	2,533 (2,135 - 2,815)
1 year cumulative dose of methotrexate in mg	455 (385 - 587.5)	435 (390 - 642.5)

Table II. Inflammatory parameters (median + range).

	Pamidronate group		Placebo group	
	Before therapy	After 12 mos.	Before therapy	After 12 mos.
Ritchie index	14 (6-22)	1 (0-16)*	13 (6-19)	3 (0-12)*
Number of swollen joints	10 (2-15)	1 (0-12)*	9 (2-15)	1 (0-6)*
HAQ	28 (3-39)	7 (0-22)*	16 (2-39)	7 (0-21)*
VAS (mm)	53 (40-78)	12 (2-72)*	52 (42-76)	9 (2-64)*
DAS by physician	3 (2-4)	1 (1-3)*	3 (3-4)	2 (1-4)*
ESR (mm/h)	31 (5-80)	11 (4-25)*	60 (7-118)**	23 (4-62)*
CRP (mg/dL)	0.9 (0.4-6.3)	0.6 (0.2-2.8)*	3.7 (1.5-16.6)	0.6 (0.17-14.7)*

HAQ: Health Assessment Questionnaire; VAS: Visual Analogue Scale (mm); DAS: Disease Activity Score; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein

* $p < 0.05$ before versus after 12 months of therapy; ** $p = 0.04$ pamidronate group versus placebo group.

Table III. Bone remodelling parameters, bone mineral density and erosion score (median + range).

	Pamidronate group		Placebo group	
	Before therapy	After 12 months	Before therapy	After 12 months
Osteocalcin	7.4 (4.1-18.5)	6.6* (3.3-16.1)	10.9 (5.7-16.3)	6.8** (4.3-12.4)
Ca/cr ratio	0.10 (0.04-0.17)	0.11 (0.03-0.20)	0.07 (0.04-0.21)	0.11 (0.03-0.48)
BMD Lumb. Spine	1.121 (0.871-1.283)	1.163*** (0.951-1.337)	1.348 (0.894-1.543)	1.301** (0.907-1.500)
BMD Fem. Neck	0.648 (0.432-0.862)	0.650 (0.458-0.856)	0.791 (0.659-1.075)	0.803 (0.598-1.005)
BMD Fem. Troch.	0.760 (0.512-0.887)	0.768* (0.525-0.885)	0.793 (0.698-1.890)	0.806 (0.692-1.110)
BMD Hand	0.260 (0.150-0.300)	0.245 (0.150-0.320)	0.295 (0.220-0.430)	0.290 (0.200-0.430)
BMD MCP II-V	0.788 (0.374-0.966)	0.758 (0.414-0.901)	0.895 (0.639-1.349)	0.935 (0.541-1.418)
Modified Sharp score	11 (0-34)	7 (0-35)	15 (1-71)	14 (1-71)

* $p = 0.02$ before versus after 12 months therapy; ** $p = 0.01$ before versus after 12 months therapy; *** $p = 0.008$ before versus after 12 months therapy.

Osteocalcin (ng/mL); BMD = Bone mineral density (g/cm²).

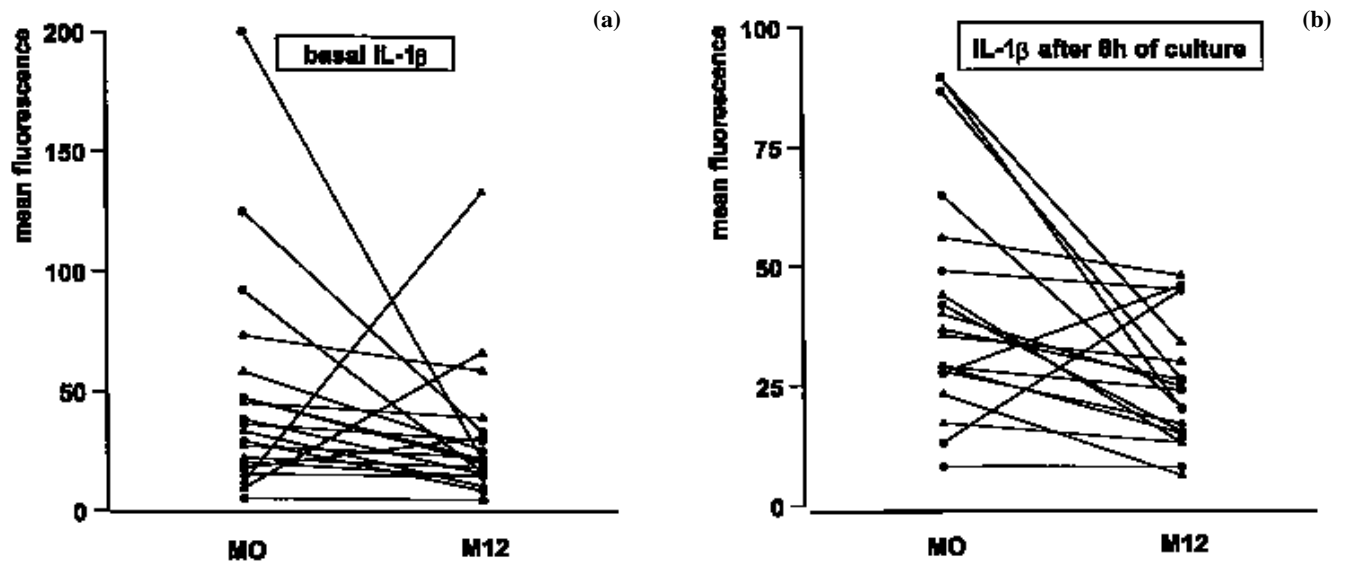


Fig. 1. IL-1 levels in patients from the pamidronate treated group ($p = 0.04$) (○) and in patients from the placebo group (□) under basal conditions (a), and after 8 hours of culture without LPS (b). MO = before therapy; M12 = after 12 months of therapy.

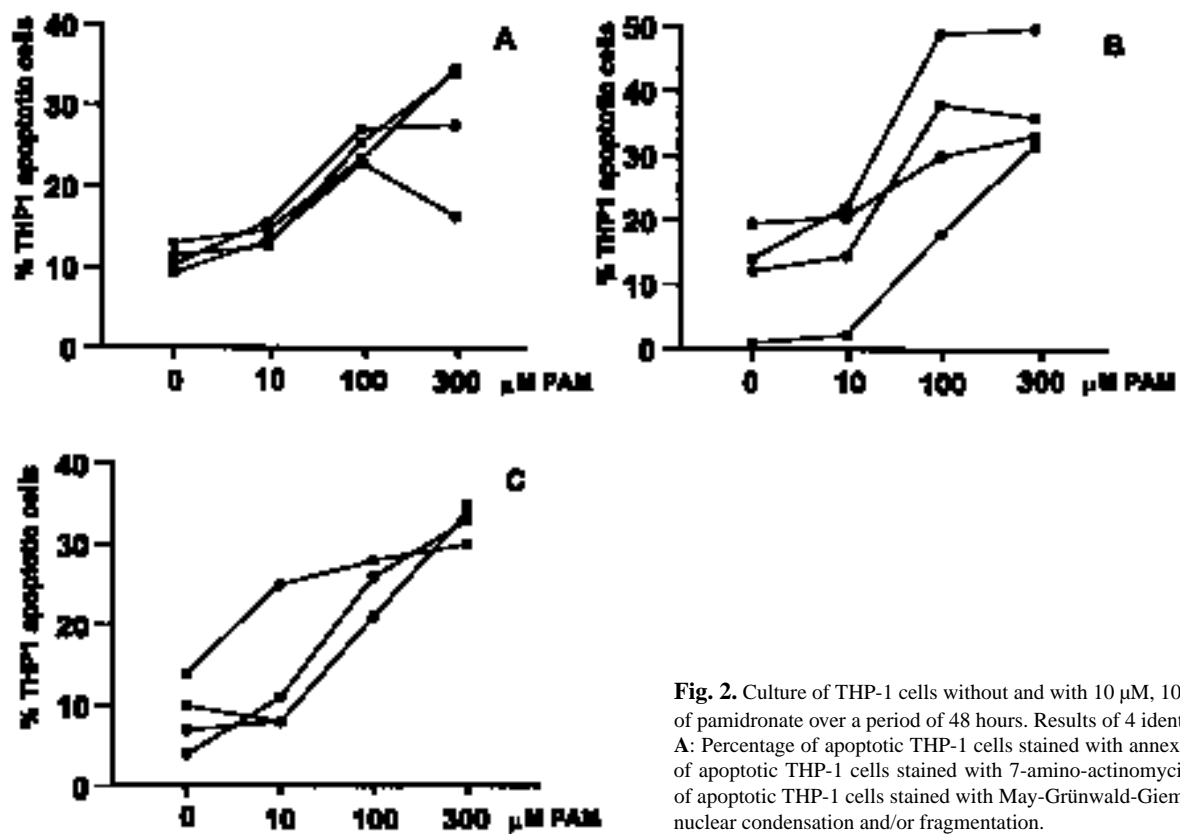


Fig. 2. Culture of THP-1 cells without and with 10 μ M, 100 μ M, or 300 μ M of pamidronate over a period of 48 hours. Results of 4 identical experiments. A: Percentage of apoptotic THP-1 cells stained with annexin; B: percentage of apoptotic THP-1 cells stained with 7-amino-actinomycin; C: percentage of apoptotic THP-1 cells stained with May-Grünwald-Giemsa showing clear nuclear condensation and/or fragmentation.

decreased in both the pamidronate group and the placebo group (respectively $p = 0.02$ and $p = 0.01$). There was no significant change in the calcium/creatinin ratio for both groups (Table III). In the pamidronate group, there was an increase in bone mineral density of the lumbar spine ($p = 0.008$) after 12 months of therapy (Table III). In the

placebo group there was a significant decrease in lumbar bone density ($p = 0.01$). The median gain was +3.8% (range: +0.5 to +12.5) in the pamidronate group versus -2.5% (range: -9.9 to +1.5) in the placebo group. The femoral neck and hand (global and metacarpal II, III, IV and V) bone mineral densities and radiological damage

scores of the hand did not change after 12 months therapy in either the placebo or the pamidronate treated groups.

Cytokines

Basal measurement of IL-1 (Fig. 1a) and spontaneous IL-1 production after 8 hours of culture (Fig. 1b) were lower after 12 months of therapy when

both treatment groups were taken together ($p = 0.02$ and 0.001 , respectively). When analysed separately, only the patients treated with pamidronate showed a statistically significant reduction in the spontaneous production of IL-1 after 8 hours of culture ($p = 0.006$). LPS-stimulated IL-1 production was not significantly altered after 12 months. There were no differences found in basal IL-6 and TNF- measurements after 12 months of therapy. Neither was there an alteration of spontaneous or LPS-stimulated production of IL-6 and TNF- in monocytes after 8 hours of culture.

In vitro apoptosis by pamidronate

The results of 4 identical experiments on THP-1 cells are shown in Figure 2. There was a progressive increase in the percentage of annexin-V-positive and propidium iodide-negative cells (apoptotic) in response to treatment with pamidronate (10-100-300 M), ranging from 13% after incubation with 10

M to 35% after incubation with 300 M pamidronate (Fig. 2A). 7-AAD staining revealed a progressive increase in the number of apoptotic hypodiploid cells (Fig. 2B): the hypodiploid cells were detectable (2-22%) after incubation with 10 M pamidronate and reached a maximum (31-50%) after incubation with 300 M pamidronate. Only in one of the four experiments did the highest concentration (300 M) result in a significant decrease in the viability of the cells. When analysed by light microscopy, the pamidronate treated cultures also showed a dose-dependent increase in the number of cells with apoptotic bodies (Fig. 2C), ranging from 8% (10 pM pamidronate) to 35% (300 M pamidronate) of apoptotic THP-1 cells.

Discussion

There is a well-known association of osteoporosis with rheumatoid arthritis (1-4). Juxta-articular osteoporosis is one of the earliest radiological abnormalities in RA (13) and hand bone densitometry correlates inversely with functional impairment (HAQ score), disease duration and radiological damage (14, 15). Generalised bone loss

seems to be related not only to functional impairment but also to disease activity and disease duration (16, 17).

In this study, the treatment of 20 rheumatoid arthritis patients with a low dose of prednisolone in combination with methotrexate resulted in a significant reduction in the clinical and biological parameters of disease activity after one year. Corticosteroids represent an important treatment for active rheumatoid arthritis, but there is a dose-related risk for steroid induced osteoporosis. In combination with disease-modifying anti-rheumatic drugs such as methotrexate, a lower dose can be used for longer periods of time. Although still a matter of debate, some studies have suggested a protective effect of treatment with low dose prednisolone on the progression of joint destruction (18, 19). Moreover, steroids increase mobility in rheumatoid arthritis and thereby reduce one of the well-known risk factors for systemic osteoporosis (5).

Bisphosphonates may be useful in the prevention and treatment of steroid induced osteoporosis (6, 7). This was also demonstrated for intermittent intravenous pamidronate (20). Furthermore, oral pamidronate was previously shown to increase bone mass in rheumatoid arthritis (21). In this study, despite treatment with steroids and the suppression of bone formation as measured by osteocalcin, the patients treated with intermittent intravenous pamidronate showed a significant rise in bone density in the lumbar spine, as opposed to a significant decline in the placebo group.

Bisphosphonates are strong inhibitors of bone resorption. Their mode of action has still not been completely elucidated, but direct effects on osteoclast metabolism are involved. Loss of protein prenylation could account for many of the effects of nitrogen-containing bisphosphonates on osteoclast morphology, resorption activity and apoptosis (22). Osteoclasts originate from hematopoietic stem cells and belong to the monocyte/macrophage cell family. In the *in vitro* part of this study, a dose-related *in vitro* apoptosis was induced in a human monocytic cell line by

pamidronate. Others have shown that bisphosphonates can induce apoptosis in macrophages (23, 24) and in human myeloma cell lines (25).

Although the etiology of rheumatoid arthritis is poorly understood, there is general agreement on the role of highly activated monocytes/macrophages in the pathophysiology of this disease. In the inflamed synovium, activated monocytes/macrophages have been shown to produce large amounts of pro-inflammatory cytokines such as IL-1, TNF and IL-8 (26). IL-1 and TNF induce osteoclast mediated bone resorption and can contribute to the more generalised osteoporosis seen in rheumatoid arthritis (4). Both IL-1 and TNF are considered to be major mediators of joint destruction and pannus formation (27). Greater numbers of macrophages in the rheumatoid synovial membrane correlate with a worse radiological outcome (28) and serum levels of IL-1 may correlate with the development of joint erosions (29). Although some experimental evidence exists in the literature (30), it is not clear whether bisphosphonates can really prevent joint destruction. In our study radiological damage scores did not change after 12 months in either the placebo or the pamidronate group, but the number of patients studied was small.

In this study, the clinical and biological parameters of disease activity were lower after 12 months of therapy with steroids and methotrexate. This might reflect the general efficiency of this regimen in the treatment of inflammation. There are scarce reports suggesting an anti-inflammatory property of bisphosphonates in experimental arthritis (31-33). In one clinical study a single infusion of pamidronate reduced disease activity in patients with rheumatoid arthritis (34). Although aminobisphosphonates can induce an acute phase response possibly related to a release of proinflammatory cytokines (35, 36), in this study we investigated whether chronic exposure to these compounds could lead to a depletion of intracellular cytokine production in monocytes. When both treatment groups in this study were analysed separately, the reduced production of IL-

1 by peripheral blood monocytes was only statistically significant in the group treated with pamidronate, confirming an additional effect of pamidronate on the suppression of inflammation besides the effect of steroid and methotrexate therapy. This seems to be confirmed by a recent placebo controlled trial where a significant decrease in serum IL-1, IL-6 and TNF- α was observed in patients with rheumatoid arthritis treated with oral alendronate for 90 days (37). A possible mechanism of this anti-inflammatory effect of pamidronate on monocytic cytokine profiles could be the induction of the apoptosis of monocytes, as demonstrated in this study on the monocytic THP-1 cell line.

In conclusion, this double-blind randomised prospective study demonstrated that pamidronate therapy resulted in an increase in bone density despite treatment with steroids. This rise was associated with a suppression of interleukin-1 production in the monocytes of patients treated with pamidronate. Our *in vitro* experiments suggest that this anti-inflammatory effect could be due to an increase in the apoptosis of monocytic cells.

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