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Colchicine induces membrane-associated activation of matrix metalloproteinase-2 in osteosarcoma cells in an S100A4-independent manner

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Abstract

Like the metastasis-associated protein S100A4, matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) are important in physiological and pathological conditions. Previously, we showed that S100A4 is involved in the regulation of MMPs and TIMPs, and in the present work we have investigated whether the anti-inflammatory and microtubule-disrupting drug colchicine has an effect on the expression of these proteins in osteosarcoma cell lines (OHS) with high and low levels of S100A4. Colchicine treatment of the various OHS cells resulted in an increased expression of MT1-MMP and TIMP-2 mRNA, and a corresponding increase of these two proteins in isolated cell membranes. Colchicine-treated cells produced more of the activated form of MMP-2 than control cells. However, the drug did not affect the amount of MMP-2 and TIMP-1 mRNA or protein, and it reduced the S100A4 mRNA expression. Isolated cell membranes from the colchicine-treated cells were more effective in activating exogenous proMMP-2 than membranes from control cells, and inhibitory studies indicated that it was the colchicine-induced increase in MT1-MMP that caused the increased activation of endogenous MMP-2. A peptide inhibitor of nuclear factor κB nuclear translocation, SN50, blocked the colchicine-induced activation of proMMP-2 and reduced the synthesis of MMP-2 in colchicine-treated cells, but not in control cells. It can be concluded that colchicine modulates the expression of MT1-MMP and TIMP-2 and hence the activation of proMMP-2 independently of the S100A4 level in osteosarcoma cells.

Keywords: S100A4; Matrix metalloproteinases; Inhibitors of matrix metalloproteinases; Cytoskeleton; NF-κB

1. Introduction

MMPs constitute a family of zinc enzymes which are involved in the degradation of extracellular matrix components as well as non-matrix proteins [1–3]. Both soluble and membrane bound forms of MMPs have been characterized [4]. These enzymes consist of a signal peptide, a propeptide, a catalytic and a hemopexin-like domain. Most of the soluble MMPs are secreted as proenzymes that can be activated by other proteinases, such as trypsin, thrombin, or plasmin, as well as by other MMPs [5]. *In vitro*, the zymogens can also be activated by organomercurials, detergents or chaotropic agents [1,5]. Regulation of MMP-2 activity is mainly achieved by activation of the pro-form, and it is assumed that the membrane bound MMPs (MT-MMPs) are the physiologically most important proMMP-2 activators [4,6,7]. At low concentrations, tissue inhibitor-2 of matrix metalloproteinases (TIMP-2) is a positive modulator of the MT-MMP-induced activation of proMMP-2, while high concentrations inhibit the

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Abbreviations: MMPs, matrix metalloproteinases; MT-MMPs, membrane-type MMPs; TIMPs, tissue inhibitors of MMPs; FCS, fetal calf serum; Mca-PLGLDpaAR, (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-[*N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]Ala-Arg-NH_2; APMA, *p*-aminophenylmercuric acetate; NF-κB, nuclear factor κB; NEM, *N*-ethylmaleimide.

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activation process by blocking the MT-MMP activity [8,9]. MT-MMPs are activated intracellularly by furin [10,11] and extracellularly by plasmin [12]. The activity of MMPs is also regulated by endogenous inhibitors, such as tissue inhibitors of matrix metalloproteinases (TIMPs) and α2-macroglobulin [1,13]. As reviewed recently, MMPs have a complicated biological function [3], playing a sophisticated role in modulating normal cellular behavior, cell–cell communication, and tumor progression. The MMPs are associated with a variety of normal and pathological conditions that involve matrix degradation and remodeling.

S100A4 is a small calcium binding protein, and has been found at higher levels in malignant than in benign tumor cells [14]. Both *in vitro* and *in vivo* experiments performed in order to study the biological effects of S100A4 have demonstrated a link between the expression of the protein and the invasive and metastatic capacity of cells [14]. Recently, we have studied the role of the metastasis-associated protein S100A4 in the metastatic process. We used an osteosarcoma cell line transfected with a vector containing a ribozyme that cleaves S100A4 mRNA, and as a control, the cell line was transfected with the vector alone [15]. Our previous studies demonstrated that S100A4 is involved in the regulation of MMP and TIMP expression in a cell density-dependent manner, whereas the S100A4-induced activation of proMMP-2 is independent of cell density [16]. Thus, at low cell density the S100A4-induced activation of proMM-2 is regulated through the nuclear factor κB (NF-κB) pathway, while this is not the case in confluent cell cultures [16]. It has previously been shown that a fraction of the NF-κB/IκBα complex can be located to microtubules and that microtubule depolymerizing agents, such as nocodazole and colchicine, induces NF-κB activation [17–19]. It has also been suggested that S100A4 interacts with microtubules resulting in microtubule dissolution [20]. Moreover, several studies have indicated that the morphology of the actin cytoskeleton is of major importance for the cellular activation of endogenous proMMP-2, while the morphology of the microtubule network is unimportant [21].

Disruption and reorganization of the microtubule network by agents, such as colchicine, colcemide, and nocodazole, have an effect on cell morphology, as well as on the integrity and expression of cellular and extracellular proteins. Due to the effect on the microtubules, these compounds arrest cells in mitosis and affects other microtubule-dependent functions [22]. Colchicine has been shown to alter the expression of MMPs and other proteins in a variety of cells and tissues [23–25]. Because of its anti-fibrotic, anti-mitotic, and anti-inflammatory activities, colchicine has been therapeutically used in the treatment of various diseases, such as gout, cirrhosis, sclerosis, Behcet's syndrome, Mediterranean fever, and Sweets syndrome [26]. Colchicine is also reported to have an anti-metastatic effect in tissue culture and in tumor-transplantation experiments in animals, and has been used with positive outcome in clinical trials on various cancer forms [27–29]. On the other hand, the therapeutic effect of colchicine has in many cases been questioned, and in some cases colchicine treatment has resulted in severe side effects [26].

In the present work, we have investigated the effects colchicine has on the expression of MMPs and TIMPs from osteosarcoma cells (OHS) with a high and a low level of the metastasis-associated protein, S100A4. The rationale for this study is manifold. (1) As colchicine has been reported to be anti-metastatic and tested as an anti-tumor drug, it was of interest to investigate its *in vitro* effect on some known factors involved in tumor progression and metastasis. (2) Is its *in vitro* effect the same on a highly metastatic cell line with a high level of S100A4 as its effect on a cell line with a largely reduced metastatic capacity due to a reduced S100A4 level? (3) In fibroblasts it has been reported that the morphology of the actin cytoskeleton, but not the microtubule cytoskeleton, is important for the ratio of active MMP-2 to proMMP-2. As dissolution of the microtubule network is reported to induce actin stress fibers, what effect does colchicine have on the ratio of active MMP-2 to proMMP-2 in the OHS cell lines with various S100A4 levels? (4) As dissolution of the microtubule network is reported to result in NF-κB activation, will a specific NF-κB inhibitor have an effect on the colchicine-induced changes seen in the OHS cells?

2. Materials and methods

2.1. Materials

DME containing HAM's F12 medium, fetal calf serum (FCS), L-glutamine, non-essential amino acids, Trizol, penicillin, streptomycin, and Geneticin (G418) were from Gibco, Life Technologies, Inc., and the serum replacement RenCyte from MediCult AS. The ELISA kits, the primary antibodies used for Western blotting and purified human TIMP-1 and TIMP-2 were from Amersham. Secondary antibodies, colchicine, α-tubulin antibodies, gelatin (Bloom 300), 1,10-phenanthroline, EDTA, *N*-ethylmaleimide (NEM), pepstatin, leupeptin, and BSA were from Sigma Chemical Co. Pefaboc was from Pentapharm LTD. Alexa™ 594-conjugated phalloidin and Alexa™ 488-conjugated secondary antibody were from Molecular Probes, Inc. The polyvinylidene difluoride membranes used for Western blotting were from Millipore Corp. and the CDP-Star chemiluminiscence substrate from New England Biolabs. Purified human proMMP-2 was from Chemicon International, Inc., and MMP-2 substrate from Bachem AG. Human fibroblast TIMP-2, the peptide inhibitor for NF-κB, SN50, and the control mutant peptide, SN50M, were from Calbiochem. Human recombinant TIMP-1 was from Oncogene. The S100A4 polyclonal antibody was from Dako A/S.

2.2. Cell cultures

The OHS cell line was established from a bone tumor biopsy from a patient treated at the Norwegian Radium Hospital and grown as monolayer cultures [30]. The cells were transfected with a vector containing a hammerhead ribozyme directed against the metastasis promoting gene S100A4 as previously described [31]. The transfectants were subcultivated in basal medium (DME containing HAM'S F12 medium, 20 mM HEPES, pH 7.2–7.4, 400 μg/mL Geneticin, 2.0 nM L-glutamine, non-essential amino acids (100× dilution), penicillin 100 IU/mL, and streptomycin 100 μg/mL) containing 10% FCS. The cells were kept in a humidified 5% CO_2 atmosphere at 37°. The ribozyme-transfected clones were designated II-11a and II-11b, whereas the control cell line, which was transfected with the vector alone, was designated pHβ-1.

2.3. Cell morphology

2.3.1. Immunocytochemistry

130,000 cells were seeded per 2 cm^2 well in basal medium containing 10% FCS and allowed to settle overnight. The cells were fixed by adding a freshly made solution of paraformaldehyde directly to the medium (final concentration of 4%) and permeabilized for 10 min with 0.5% Triton X-100 before incubation with 3% BSA in PBS (blocking solution) for 30 min. For actin staining, fluorescent phalloidin (Alexa 594-conjugated) was added in a 1:40 dilution for 30 min. For tubulin staining the cells were incubated with anti-α-tubulin antibodies in a 1:100 dilution for 30 min. The immunostaining was developed using an Alexa 488-conjugated goat anti-mouse IgG secondary antibody diluted 1:1000 and analyzed by fluorescence microscopy.

2.3.2. Preparation and Northern blot analysis of RNA

Total cellular RNA was prepared from confluent cells that were either treated or not treated with colchicine. RNA was extracted with Trizol according to the manufacturer's instructions. Samples of 5 μg were separated by 1% agarose–formaldehyde gel electrophoresis, and the filters were hybridized with DNA probes labeled with ^{32}P by the random priming technique [32]. The hybridizations were carried out in 0.5 M sodium phosphate (pH 7.2), 7% SDS, and 1 mM sodium EDTA at 65° [33]. The membranes were subsequently washed three times for 15 min in 40 mM sodium phosphate (pH 7.2) and 1% SDS at 65°. To calibrate the filters and correct for uneven amounts of RNA loaded in each lane, the filters were hybridized by a kinase-labeled oligonucleotide probe complementary to nucleotides 287–305 in human 18S rRNA as described previously [15]. Images obtained in a STORM Phospho Imager (Amersham Biosciences) were used to determine the signal intensities. The mRNA expression levels were calculated relative to the expression of 18S rRNA. For each cell line treated with or without colchicine, two to four independent samples were analyzed, and the mean expression level of a particular mRNA was related to the mean expression level of the untreated controls.

The following probes were used: (a) the cDNA probe for S100A4, kindly provided by Dr. E. Lukanidin, the Fibiger Institute, Copenhagen, Denmark [34]; (b) the cDNA probes encoding TIMP-1 and TIMP-2, kindly provided by Dr. W.G. Stetler-Stevenson [35]; (c) the human cDNA for MT1-MMP, a generous gift from Dr. H. Sato [6]; and (d) the cDNA for MMP-2, a kind gift from British Biotech Pharmaceuticals.

2.4. Production of conditioned media for MMP and TIMP determination

To determine the secretion of MMPs and TIMPs into the culture medium, 0.3, 0.6, 1.2, 1.9, 2.4, and 4.8 million cells were seeded in 25 cm^2 culture flasks in basal medium containing 10% FCS. After overnight cultivation, the cells were washed three times with serum-free basal medium containing 1.0 mg/mL of the non-protein serum replacement, RenCyte. Colchicine in concentrations of 0.1, 0.5, 1.0, and 2.0 μM was added to the serum-free basal media, and the cultures were subsequently maintained for 48 hr in 4 mL of this serum-free basal medium containing colchicine. Cell cultures in serum-free media without colchicine were also maintained for 48 hr, as controls. Prior to freezing the harvested media was made 10 mM in $CaCl_2$ and 100 mM in HEPES, pH 7.5. Subsequently, the cells were trypsinized and counted.

2.5. Effect of SN50 and SN50M on colchicine-induced activation proMMP-2

pHβ-1 cells (0.38 million per 2 cm^2) were seeded in 24-well plates in basal medium containing 10% FCS, and were allowed to settle overnight. The cells were then washed three times with serum-free basal medium containing RenCyte as described above, and then incubated for 48 hr in 350 μL of this RenCyte containing serum-free medium with and without 1.0 μM colchicine. In addition, the serum-free medium contained 0, 50, and 100 μg/mL SN50 or SN50M. The conditioned media were collected as described above and analyzed by gelatin zymography.

2.6. Isolation of cell membranes

Approximately 3×10^7 cells were seeded in 150 cm^2 culture flasks, and allowed to settle for 48 hr in basal medium containing 10% FCS. The cells covered the entire culture area, resulting in a confluent cell layer. Thereafter, the cells were washed three times with serum-free basal medium containing 1.0 mg/mL non-protein serum replacement, RenCyte. The cultures were subsequently maintained for approximately 38 hr in 20 mL of the same serum-free basal medium with or without 1.0 μM colchicine. Plasma

membranes were prepared as previously described [9]. Briefly, the cells (1×10^8 to 3.5×10^8) were scraped off, suspended in 20 mM Tris–HCl, pH 7.4, 8.7% (w/v) sucrose and pelleted at 5900 *g*. The cell pellet was re-suspended in the same buffer, homogenized and thereafter sonicated 10 times for 10 s on ice at an intensity of 5. The homogenate layered on top of a 38.5% (w/v) sucrose cushion prior to centrifugation (100,000 *g*, 1 hr). The cell membrane fraction was collected from the interphase, pelleted by centrifugation (100,000 *g*, 1 hr), and suspended in 50 mM Tris–HCl, pH 8.0, 5 mM $CaCl_2$. All steps were performed at 4°. The membrane preparation was frozen at −20°. The amount of total membrane protein was determined using the Bradford method (BioRad) with BSA as the standard.

2.7. Gelatin zymography

SDS–substrate PAGE was performed as described previously [36], with gels (7.5 cm × 8.5 cm × 0.75 mm) containing 0.1% (w/v) gelatin in the separating gel, which was either 7.5 or 10% in polyacrylamide. Calibration of the zymograms was performed as follows. In some cases standard protein markers of M_r 20,000–200,000 were used. In other cases, we used mono (92 kDa) and dimer (225 kDa) forms of MMP-9 from serum-free culture medium of THP-1 cells [37], and 72-kDa MMP-2 from serum-free culture medium of human skin fibroblasts [36]. We also used purified 72-kDa MMP-2 or *p*-aminophenylmercuric acetate (APMA)-activated MMP-2 (62 kDa) as standards. Twelve microliters of undiluted or diluted conditioned medium was mixed with 3 μL of loading buffer (333 mM Tris–HCl, pH 6.8, 11% SDS, 0.03% bromophenol blue, and 50% glycerol). Six microliters of this non-heated mixture was applied to the gel. Thereafter, the gel was run at 20 mA per gel at 4°. After electrophoresis the gels were washed twice in 50 mL of 2.5% (v/v) Triton X-100, and then incubated in 50 mL of assay buffer (50 mM Tris–HCl, pH 7.5, 5 mM $CaCl_2$, 0.2 M NaCl, and 0.02% Brij-35) for approximately 20 hr at 37°. Gels were stained with 0.2% Coomassie brilliant blue R-250 (30% methanol) and destained with an aqueous solution of 30% methanol and 10% acetic acid. Gelatinolytic activity was evident as transparent zones in the blue gels. The area of the cleared zones was analyzed with the GelBase/GelBlot™ Pro computer program from Ultra Violet Products.

2.8. Reverse gelatin zymography

Gelatin was labeled with the fluorescent dye 2-methoxy-2,4-diphenyl-3(2*H*)-furanone to give MDPF-gelatin as described previously [38]. MMP inhibitory activity was assayed by electrophoresis in polycacrylamide gels containing the fluorescent MDPF-gelatin as MMP substrate and PMA-stimulated THP-1 monocyte culture-conditioned media as a source of MMP. The predominant MMP in this media is the monomeric form of MMP-9, and in addition, it contains TIMP-1 [37]. Briefly, we used 75 mm × 85 mm × 0.75 mm gels, where the separating gel was 13% in polyacrylamide, 0.1% (w/v) in MDPF-gelatin, and 2% (v/v) in THP-1 culture-conditioned media. Prior to loading the samples onto the gel, a pre-run of the gel was performed for approximately 2 hr at a constant current of 14 mA per gel, at 4°. This was done in order to remove the TIMP-1 in the THP-1 medium, which in the absence of a pre-run prevented the detection of inhibitors with a molecular size of less than 30 kDa. The zymograms were calibrated with mono (92 kDa) and dimer (225 kDa) forms of MMP-9 and TIMP-1 from serum-free culture medium of PMA-stimulated THP-1 cells, trypsin (20 kDa) and recombinant human TIMP-1 (28 kDa). Samples were treated as described for gelatin zymography, and after the electrophoresis, the gel was washed and incubated as described for gelatin zymography. However, as the method detects the disappearance of the fluorescence-labeled gelatin, it was possible to follow the reaction by UV detection at appropriate time intervals without staining the gel, and therefore this method has been called real-time reverse zymography [39]. The presence of inhibitors appears as light fluorescence bands due to a reduced degradation of the labeled gelatin. When a sample contains higher concentrations of a gelatin degrading proteinase than that incorporated in the gel, a dark band appears due to the faster degradation than of the surrounding gelatin substrate. However, the higher the degree of degradation of the surrounding gelatin, the lower the visibility of these dark bands. Thus, by finely tuning the balance of the incorporated gelatinase and by following the degradation over time, it is possible to detect both MMP and MMP inhibitors in the same gel.

2.9. Western blot analysis

Conditioned media and isolated cell membranes from pHβ-1 and II-11b cells were electrophoresed on SDS–polyacrylamide gel (4% (w/v) in stacking gel and 7.5% (w/v) in separating gel) and transferred to a polyvinylidene difluoride membrane according to the manufacturer. After blockage of non-specific binding sites with non-fat milk (5% solution), blots were incubated for 1 hr at room temperature with primary mouse monoclonal antibodies either against MMP-2, MT1-MMP or TIMP-2. After washing, the blots were incubated for 1 hr at room temperature with an alkaline phosphatase-conjugated secondary antibody diluted 1:20,000 in blocking solution, and developed with CDP-Star™ chemiluminiscence substrate according to the description of the manufacturer.

2.10. Assay of MMP-2 activity

The activity of MMP-2 was determined fluorometrically at 30° using (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-[*N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-

Ala-Arg-NH_2 (Mca-PLGLDpaAR) as substrate [40,41]. The assay solution consisted of 5.0 μM substrate in 0.1 M HEPES, pH 7.5, 10 mM $CaCl_2$, and the initial reaction velocity was recorded using a Perkin-Elmer LS 50B spectrofluorometer (0.5-cm light path cuvettes) by excitation at 323 nm and continuously measuring emission at 393 nm.

2.11. Activation of purified proMMP-2 by plasma membranes

Membrane-mediated activation of purified human proMMP-2 was performed by incubating the proenzyme (3 μg/mL; 42 nM) with 5.0 μg membrane protein in 50 mM Tris–HCl, pH 8.0, 5 mM $CaCl_2$ for up to 22 hr at 37°. Each pre-activation mixture was analyzed by gelatin zymography, and for that purpose an aliquot was withdrawn at appropriate time intervals and diluted so that each well in the zymography gel contained a total amount of 1.7 ng of MMP-2 and 79 ng of membrane protein. In other experiments, the activation was followed by kinetic assays, using the chromogenic substrate Mca-PLGLDpaAR (5 μM) in 0.1 M HEPES, pH 7.5, 10 mM $CaCl_2$ at 30°, with 50 ng MMP-2 in each assay. To determine if known metallo, serine, thiol, or acid proteinase inhibitors have an effect on the activation, isolated membranes from colchicine-treated pHβ-1 cells were mixed with proMMP-2 (as described above) in the presence of either 10 mM EDTA, 1 mM 1,10-phenanthroline, 1 mM Pefabloc, 1 mM NEM, 2 μg/mL Leupeptin, 1 μg/mL Pepstatin, 1.5 μg/mL TIMP-1, or 15.5 μg/mL TIMP-2.

2.12. Statistics

Statistical analyses were performed using the Student's *t* test for independent analysis to evaluate differences between the cell lines with respect to expression of MMPs and TIMPs. Data are presented as mean ± SD (Northern blots, gelatin zymography, and Western blotting data). A *P* value of 0.05 was considered significant. Analyses were based on three or more independent cell culture experiments for each cell line, density, and colchicine concentration. Conditioned media from each of these experiments were run in triplicate on gelatin zymography and in duplicate on ELISA and Western blots.

3. Results

3.1. Morphology and cytoskeletal structure

Investigation of cell morphology by light microscopy showed that the untreated pHβ-1 cell line exhibited a more rounded cell shape than the II-11 cell lines at any of the cell densities investigated (not shown), which is in agreement with previous observations [31]. Treatment with colchicine resulted in rounded cells and reduction of adhesive properties (data not shown). The structure of the actin cytoskeleton and the microtubules in colchicine-treated as well as in untreated pHβ-1 and II-11 cell lines were investigated by immunofluoroscence microscopy. In the untreated cell lines, there were no differences between the cell lines with respect to the morphology of either the actin or the tubulin cytoskeleton (Fig. 1). In the presence of colchicine, the microtubules were dissolved, while the actin cytoskeleton remained unchanged (Fig. 1). The colchicine-induced changes in tubulin morphology were observed in all the examined cell lines, suggesting that this process is independent of the level of S100A4 expression.

3.2. The effect of colchicine on the cell lines expression of specific mRNA species

The Northern blots in Fig. 2 show the mRNA expression of 18S, S100A4, MMP-2, MT1-MMP, TIMP-1, and TIMP-2 in colchicine-treated and untreated confluent pHβ-1 and II-11a cells. The diagrams on the right show the relative expression of the various transcripts after normalization against the 18S levels. The same trends as in Fig. 2 were obtained in other independent experiments where the effect of colchicine (0, 1.0, and 2.0 μM) was investigated on pHβ-1 and II-11b cells (data not shown).

The II-11a cells displayed about a 90% reduction in S100A4 expression as compared to the pHβ-1 control cells (Fig. 2), confirming the ribozyme-induced reduction in S100A4 mRNA level. As also shown in Fig. 2, colchicine treatment resulted in an approximately 35% reduction in the S100A4 level in the pHβ-1 cells. Colchicine induced the same response in the II-11a, but due to the already low amount of S100A4 in the untreated cells it was impossible to quantitatively determine this reduction.

Colchicine treatment resulted in an up-regulation of MT1-MMP and TIMP-2 mRNA in both cell lines (Fig. 2). However, as the level of S100A4 apparently had no effect on the colchicine-induced up-regulation of MT1-MMP and TIMP-2 mRNA levels, the pooled results (mean ± SD and N = 4) for the two cell lines at colchicine concentrations of 0, 0.5, and 1.0 μM were as follows: MT1-MMP, 100 ± 13, 249 ± 24, $P = 3.9 \times 10^{-5}$ and 290 ± 75, $P = 2.5 \times 10^{-3}$; TIMP-2, 100 ± 15, 227 ± 33, $P = 4.3 \times 10^{-4}$ and 232 ± 40, $P = 8.2 \times 10^{-4}$. In contrast to this, colchicine did not alter the level of MMP-2 or TIMP-1 mRNA in either of the two cell lines (Fig. 2), and the apparent small increase in TIMP-1 mRNA in both cell lines was not significant (confidence level of 88% as analyzed on pooled results).

3.3. The effect of colchicine on the synthesis of soluble MMP and TIMP proteins

It was previously shown that colchicine increased the synthesis of MMP-1 from human skin fibroblasts, and this increase was dependent on both the concentration of

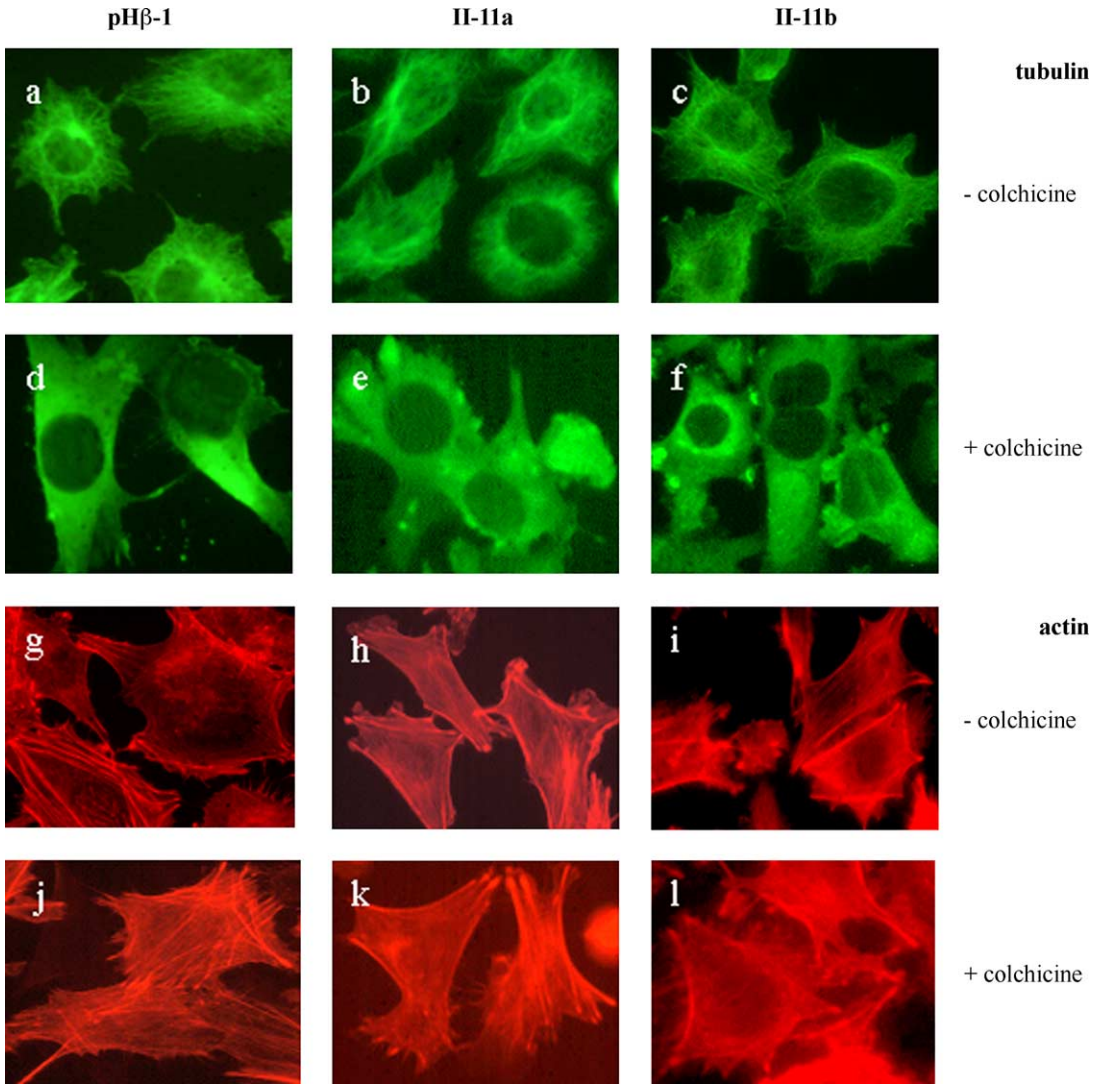

Fig. 1. Morphology of the actin and tubulin cytoskeleton in osteosarcoma cells with a high and a low S100A4 level. Osteosarcoma cell lines with a high (pHβ-1) and a low (II-11a and II-11b) S100A4 expression were seeded at 130,000 cells per well in 24-well plates, cultured for 24 hr in medium containing 10% FCS. Thereafter, the cells were incubated for 6 hr in serum-free DME-F12 medium with or without 1 μM colchicine, in addition to the additives as described in Section 2. Actin organization was observed by staining the cells with the F-actin probe BODIPY^{TM} phalloidin labeled with red fluorescence. For staining of the microtubules, a primary antibody against α-tubulin and a secondary antibody labeled with green fluorescence were used. In all three cell lines with (j–l) or without (g–i) colchicine treatment, the actin staining is predominantly at the cell cortex and at focal adhesions. In the three cell lines without colchicine treatment (a–c), microtubules are shown as fibrillar filaments, whereas the staining was diffuse in the colchicine-treated cells (d–f). Magnification 400×.

colchicine and on the density of the fibroblast monolayer [25]. We have investigated the ability of colchicine to modulate the MMP and TIMP protein synthesis in pHβ-1 and II-11b cells using various drug concentrations at different cell densities as described in Section 2. Conditioned media were analyzed for MMPs and TIMPs using gelatin zymography, ELISA, and Western blotting. In both cell lines and at all cell densities, gelatin zymography of harvested medium from the untreated cells revealed two main bands, one at 72 kDa and another at around 62 kDa. The 62-kDa band became more pronounced with increasing cell density for both cell lines. However, the main difference between the two cell lines was that the pHβ-1 cells produced more of the 62-kDa band than the II-11b cells at all cell densities. For both cell lines, the effect of colchicine at all concentrations and cell densities was to induce more of the 62-kDa band in relation to the 72-kDa band. A typical gelatin zymography is shown in Fig. 3 for the II-11b cells. The increase in the 62-kDa band with increasing colchicine concentrations became less evident at high cell densities for the pHβ-1 cells, as a significant amount of the 62-kDa band was already present in the untreated cells. Western blots verified that both the 72-kDa and the 62-kDa bands are MMP-2 (Fig. 4). In some experiments, a third band appeared at approximately 64 kDa. Thus, it appears that colchicine induces an activation of proMMP-2 without altering the total level of MMP-2 and that this induction is independent of both the S100A4 level and the cell density.

Most of the harvested conditioned serum-free media revealed only the 72-kDa and 62-kDa forms of MMP-2 and

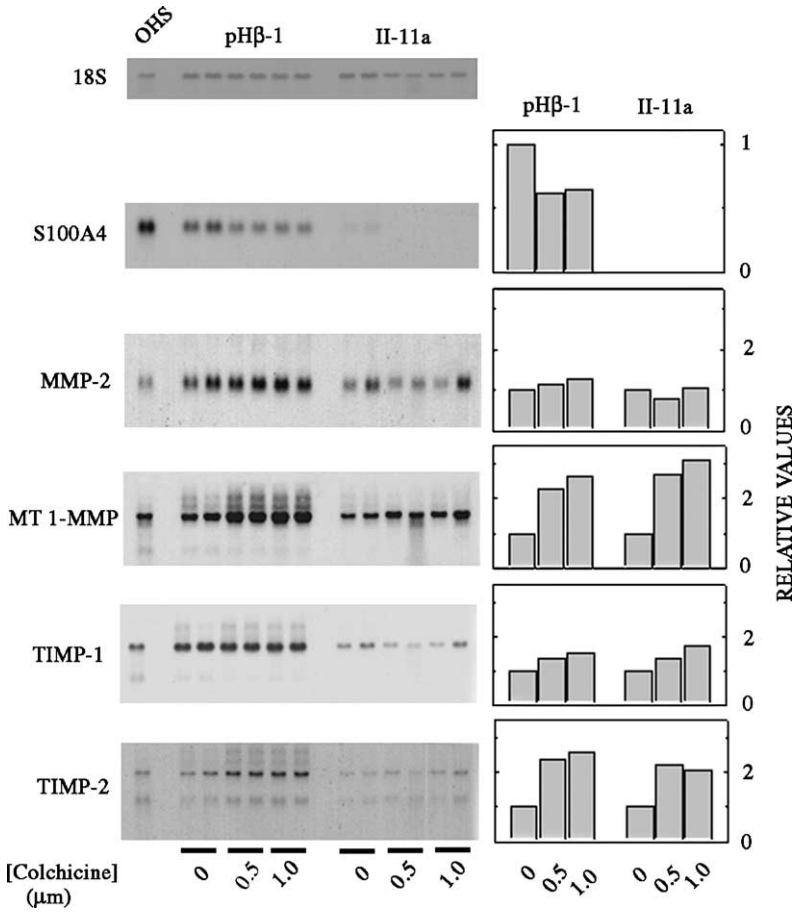

Fig. 2. Representative Northern blot analysis of S100A4, TIMPs and MMPs in colchicine-treated and untreated osteosarcoma cells. The osteosarcoma cell lines pHβ-1 and II-11a were grown and treated with colchicine according to standard incubation conditions as described in Section 2. Total RNA was extracted from confluent cell cultures. Also shown as an control is the parental cell line OHS. At the right is shown the mean expression of the various mRNA's normalized against the corresponding 18S rRNA loading control, where the colchicine-treated cells is reported relative to the amount of the untreated controls.

no other bands. Only in sporadic cases weak bands appeared with molecular sizes somewhat larger than the 72-kDa band and smaller than the 62-kDa band. Neither of the two cell lines, treated or untreated with colchicine, produced high enough levels of MMP-9 to allow detection by the ELISA method. However, both cell lines produced TIMP-1, and the level was unaffected by colchicine (data not shown).

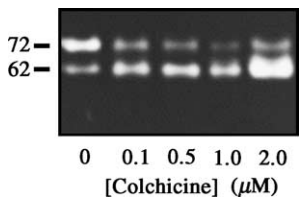

Fig. 3. Representative gelatin zymogram showing the effect of colchicine on the synthesis of gelatinases in osteosarcoma cells. The osteosarcoma cell line II-11b (1.2×10^6 cells) was seeded in 25 cm^2 culture flasks as described in Section 2. After 24 hr in serum-containing medium, cells were cultivated for 48 hr in serum-free medium containing various colchicine concentrations as shown in the figure. The harvested culture medium was analyzed on gelatin zymography as described in Section 2. The M_r markers of 72 and 62 kDa are for proMMP-2- and APMA-activated MMP-2, respectively.

3.4. MMPs and TIMPs in isolated cell membranes from control and colchicine-treated cells

MT1-MMP is expected to be the most important biological activator of proMMP-2 [4,6,7], and a fine-tuned balance of TIMP-2 modulates this membrane-associated activation [8,9]. To determine if colchicine modulates both

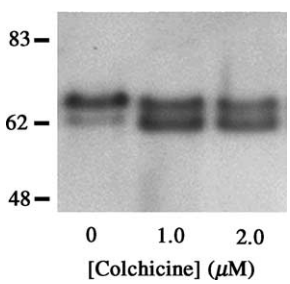

Fig. 4. Determination of MMP-2 by Western blotting. Serum-free conditioned media were obtained from II-11b cells treated with various concentrations of colchicine as described in Section 2. The blots were treated with antibodies against MMP-2 as described in Section 2. On the left is shown the position of the standard M_r markers used.

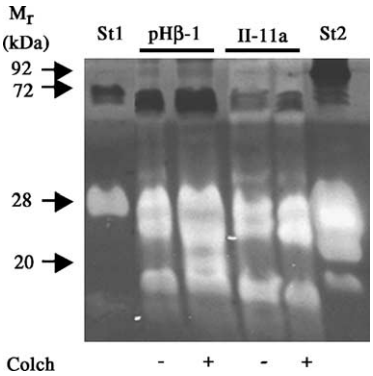

Fig. 5. Detection of gelatinase inhibitors in isolated membranes by real-time reverse zymography. Plasma membranes isolated from confluent cultures of pHβ-1 and II-11a cells cultured in the presence (+) or absence (−) of colchicine (1 μM) as described in Section 2. The following standard M_r markers were used; conditioned medium from II-11b (St1) and THP-1 cells (St2), human recombinant TIMP-1 (28 kDa, not shown), and trypsin (20 kDa, not shown). The arrow at 72 kDa shows the 72-kDa proMMP-2 in St1 and 92 kDa shows the proMMP-9 in the St2. Three micrograms of isolated plasma membranes from pHβ-1 (β) and II-11a cells (a) were loaded per well.

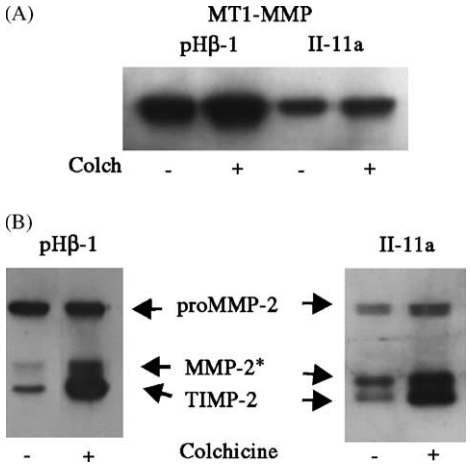

Fig. 6. MT1-MMP, MMP-2 and TIMP-2 in isolated membranes from untreated cells (−) or cells treated with 1 μM of colchicine (+). Membranes isolated from confluent cultures of pHβ-1 and II-11a cells were analyzed by Western blotting, using antibodies against MT1-MMP (A), MMP-2 (B) and TIMP-2 (B). In panel A, an M_r of 58 kDa was estimated for the MT1-MMP based on standard M_r markers. In panel B, MMP-2* shows a truncated form of MMP-2, with an M_r of 32 kDa.

the amount of MT1-MMP and TIMP-2 bound to the plasma membrane in pHβ-1 and II-11a cells, membranes isolated from colchicine-treated and control cells were analyzed by real-time reverse gelatin zymography and Western blotting. A typical reverse zymography is shown in Fig. 5, which reveals both gelatin degrading enzyme activity (dark bands) and MMP inhibitory activity (white bands) in the isolated membranes from the colchicine-treated and untreated cell lines pHβ-1 and II-11a. The membrane preparations from the pHβ-1 and II-11a cell lines show gelatin degrading bands corresponding to the position of the MMP-2 bands in the control II-11b culture medium (St1), as well as bands with somewhat lower M_r. The intensity of these bands was stronger in the membranes from the colchicine-treated cells compared to the corresponding control cells. These bands are likely to be MMP-2 bound to the membrane as well as MT1-MMP. The isolated membrane preparations also contained several inhibitory bands, three main bands at 28, 24, and 17 kDa, in addition to a weak band at 37 kDa. The 24-kDa inhibitory band was stronger in the membranes from the colchicine-treated cells than from the corresponding control cells. The M_r of this band is identical to the M_r of TIMP-2. The 28-kDa inhibitory band that corresponds to the M_r of TIMP-1 and the band at 17 kDa were not affected by the colchicine treatment. The weak band at 37 kDa was reduced in the membranes from the colchicine-treated cells.

Antibodies against MT1-MMP revealed a band at 58 kDa in all membrane preparations (Fig. 6A). A quantitative estimation revealed that there was approximately five times more of MT1-MMP in the untreated membranes from the pHβ-1 cells compared to the II-11 cells. The intensity of the band was twice as strong in the membrane preparations from the colchicine-treated cells as in the membranes from the corresponding control cells (Fig. 6A). Membranes from the colchicine-treated cells contained much more of bound TIMP-2 (24 kDa) and a processed form of MMP-2 (approximate M_r of 32 kDa) than the membranes from the control cells (Fig. 6B). The membranes from the colchicine-treated cells also contained slightly more bound proMMP-2 (72 kDa) than the membranes from the control cells (Fig. 6B).

3.5. Activation of exogenous proMMP-2 by isolated cell membranes from control and colchicine-treated cells

In order to determine whether the altered level of MT1-MMP and TIMP-2 in the colchicine-treated cells could account for the increased amount of active MMP-2 in the cell-conditioned medium, we investigated the ability of isolated membranes to activate exogenous proMMP-2. As there was much more MT1-MMP in the isolated membranes from the colchicine-treated and untreated pHβ-1 cell line compared to the II-11a cell line, we used the cell membranes from the pHβ-1 cells for this purpose. Purified proMMP-2 was incubated at 37° with membranes isolated from the control cells or the colchicine-treated cells for different time periods. Activation of the exogenously added proMMP-2 was analyzed by gelatin zymography and by using the synthetic peptide substrate Mca-PLGLDpaAR.

Prior to isolation of the membranes, cells were incubated for approximately 38 hr in serum-free medium with or without 1.0 μM colchicine. These serum-free media were collected and analyzed using gelatin zymography to ensure that colchicine had induced activation of proMMP-2. Figure 7A shows that this was the case even though more

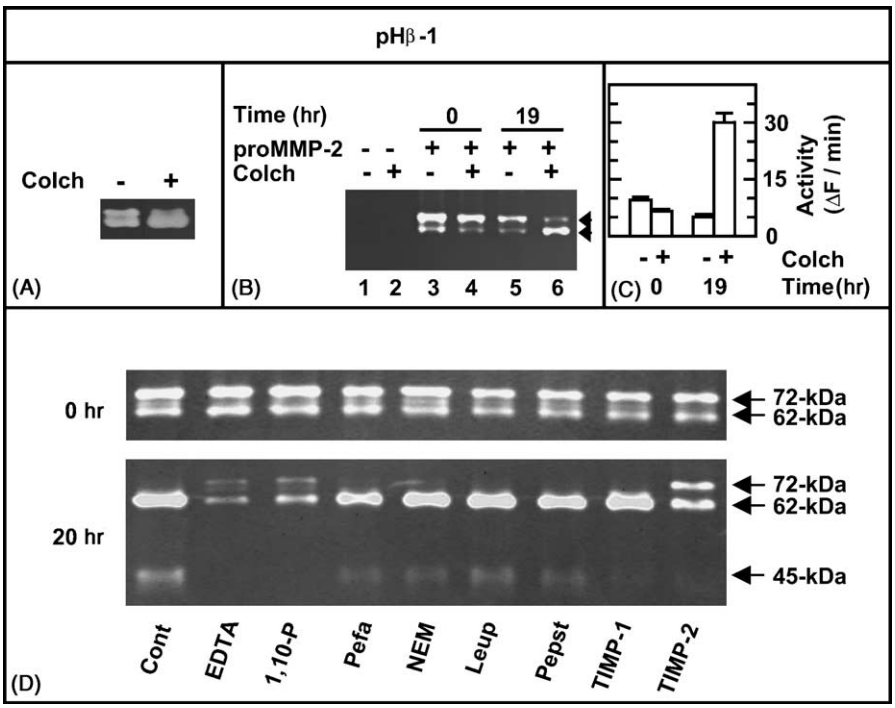

Fig. 7. Activation of proMMP-2 by isolated membranes from osteosarcoma cell lines with a high and a low expression S100A4 analyzed by gelatin zymography and activity assays. Membranes were isolated from confluent pHβ-1 cells cultured for 38 hr in serum-free cultures with or without 1.0 μM colchicine as described in Section 2. Membrane preparations were incubated with proMMP-2 as described in Section 2. (A) Gelatin zymography of the harvested serum-free media of the pHβ-1 cells showing that colchicine treatment resulted in an increased activation of the synthesized MMP-2 compared to the untreated cells. (B) Lanes 1 and 2 contain only membranes, lanes 3 and 4 contain proMMP-2 treated with membranes for 0 hr (controls), and lanes 5 and 6 contain proMMP-2 treated with membranes for 19 hr at 37°. Arrowheads at the right show the position of the 72-kDa proMMP-2 and the 62-kDa band from APMA-activated proMMP-2. (C) The proMMP-2 treated with isolated membranes was also analyzed for activity against the synthetic peptide substrate Mca-PLGLDpaAR as described in Section 2. (D) ProMMP-2 was treated with membranes in the absence (Cont) or presence of EDTA (10 mM), 1,10-phenantroline (1 mM), Pefabloc (1 mM), NEM (1 mM), Leupeptin (2 μg/mL), Pepstatin (1 μg/mL), TIMP-1 (1.5 μg/mL), and TIMP-2 (15.5 μg/mL) for 0 and 20 hr at 37°.

than 50% of the synthesized MMP-2 was in the 62-kDa form in control cells. There was also a great difference between the ability of the two isolated membrane preparations to activate exogenously added proMMP-2. Lanes 1 and 2 in Fig. 7B show that with the amount of membranes added to each lane in these activation experiments, no gelatinase bands could be detected in the two membrane preparations. In addition, no activation of proMMP-2 was observed immediately after the mixing of membranes and exogenously added proMMP-2 (Fig. 7B, lanes 3 and 4). After 19-hr incubation at 37°, the isolated membranes from the colchicine-treated pHβ-1 cells had almost totally converted the exogenously added proMMP-2 to the 62-kDa form, while almost none of the proMMP-2 was converted with the control membrane preparation (Fig. 7B, lanes 5 and 6). To verify that the conversion of the 72-kDa proMMP-2 to the 62-kDa form of MMP-2 resulted in a biologically active gelatinase, a part of the mixtures used for zymography in Fig. 7B was used to measure the enzyme activity in solution, using the synthetic peptide substrate Mca-PLGLDpaAR. As shown in Fig. 7C, only trace activities were obtained in the samples immediately after the mixing of membranes and proMMP-2 and in the control sample after 19 hr of incubation. However, after 19 hr of incubation of proMMP-2 with membranes isolated from colchicine-treated cells, the degree of activity increased significantly (Fig. 7C). This showed that the membrane-induced conversion of the 72-kDa pro-form of MMP-2 to the 62-kDa form resulted in the active form of the enzyme.

To verify that it was the increased amount of MT1-MMP in the isolated membranes from colchicine-treated cells that was responsible for the activation of the exogenously added proMMP-2, membranes were incubated with proMMP-2 in the presence of general and specific MMP inhibitors, serine proteinase inhibitors, thiol proteinase inhibitors, and an acid proteinase inhibitor. As shown in Fig. 7D, EDTA, 1,10-phenanthroline, and TIMP-2 inhibited the membrane-induced activation of MMP-2, while no effect was observed with Pefabloc, NEM, Leupeptin, Pepstatin, or TIMP-1. These results imply that it was the MT1-MMP in the isolated cell membranes that caused activation of the exogenously added proMMP-2.

3.6. Effect of SN50, an NF-κB inhibitor, on the synthesis of pro- and active forms of MMP-2 in control and colchicine-treated cells

The promotor of human MT1-MMP contains a consensus p65 NF-κB binding site [42] and microtubule dissociating

agents, like colchicine and nocodazole, are known to activate the NF-κB pathway [17–19]. Therefore, the high level of MT1-MMP and the increased activation of proMMP-2 in the colchicine-treated cells might be explained by a colchicine-induced activation of the NF-κB signaling pathway. We utilized a synthetic peptide inhibitor for NF-κB, SN50, to investigate this possibility. The inhibitor has an NF-κB nuclear translocation motif flanked by a signaling peptide that facilitates membrane permeability and blocks NF-κB translocation into the nucleus [43]. The control mutant peptide, SN50M, in which two positively charged amino acid residues are replaced, cannot block the NF-κB translocation into the nucleus. Confluent pHβ-1 and II-11b cells stimulated with 1 μM colchicine were incubated at three different concentrations (0, 50, and 100 μg/mL) of either the inhibitor or the control peptide as described in Section 2, and the harvested conditioned media were analyzed with gelatin zymography. In the untreated pHβ-1 and II-11b cells, approximately 50 and 10%, respectively, of the MMP-2 was in the active 62-kDa form and in the corresponding colchicine-treated control cells approximately 80 and 40% was in the active form (Fig. 8). In the colchicine-treated cells, the presence of SN50 reduced the active 62-kDa form in a dose-dependent manner, and at 100 μg/mL of the inhibitor the enzyme existed almost exclusively in the 72-kDa proform (Fig. 8A and C). Although SN50 reduced the amount of the active 62-kDa form in the colchicine-treated pHβ-1 to a level much lower than in the control (Fig. 8A), the presence of SN50 did not result in a reduction of the 62-kDa band in the controls (Fig. 8B). As expected, the presence of the control peptide, SN50M, did not result in a significant reduction of the active 62-kDa form of MMP-2 (Fig. 8A–C). It is also noticeable that SN50, but not SN50M, reduced the total amount of MMP-2 (i.e. the sum of the 72- and 62-kDa bands) in the colchicine-treated cells (Fig. 8A and C). These results suggest that colchicine-induced activation of the NF-κB signaling pathway resulted in the activation of proMMP-2. It also suggests that in the presence of colchicine, the synthesis of MMP-2 is at least partly regulated by the NF-κB signaling pathway.

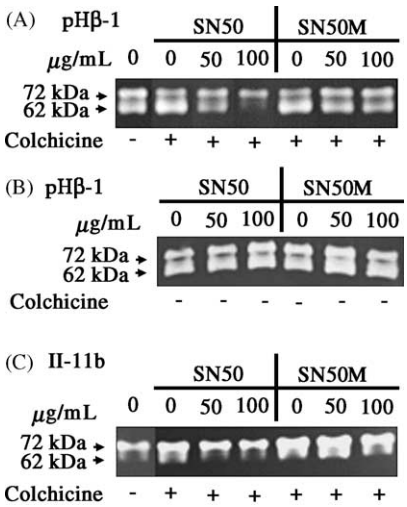

Fig. 8. Effect of the NF-κB inhibitor, SN50, on the colchicine-induced activation of proMMP-2. The cell-permeable peptide inhibitor of NF-κB, SN50, and the control mutant peptide, SN50M, were added at the indicated concentrations to confluent pHβ-1 (A and B) and II-11b (C) cells in serum-free medium with (+) or without (−) 1.0 μM colchicine as described in Section 2. Conditioned serum-free media were analyzed by gelatin zymography. Latent and active MMP-2 with molecular mass of 72 and 62 kDa, respectively, are indicated.

4. Discussion

Colchicine has previously been found to induce synthesis of collagenases and gelatinases in both fibroblasts and monocytes [23,25], as well as to down-regulate the synthesis of collagen [44,45]. In the present work, it is shown that colchicine selectively modulates the expression of various proteins in osteosarcoma cells, and that the S100A4 level in the osteosarcoma cells does not influence the effect of colchicine on the expression of these proteins. Colchicine down-regulates the S100A4 expression, up-regulates the MT1-MMP and TIMP-2 synthesis, up-regulates the activation of proMMP-2, while it has no effect on MMP-2 and TIMP-1 expression. This suggests that colchicine, which also dissolves the tubulin cytoskeleton can induce an altered regulation of various proteins, and that these effects appear to be at least partly cell-type specific.

The organization of the actin cytoskeleton has been suggested to play a major role in the activation of proMMP-2, as various agents that mediate activation of proMMP-2 also induce changes in the actin cytoskeleton [21,46–48]. Activation has appeared under conditions where the actin cytoskeleton was organized into a meshwork and lacked stress fibers [21]. It was suggested that the activation was not due to a general change in cellular morphology, as compounds that induced changes in the cell morphology by inducing rearrangements in the microtubule network did not induce activation of proMMP-2 [21]. In the present work, we show that this is not the case for the osteosarcoma cell lines studied. The control cell line pHβ-1, with a high level of S100A4, produces more of the activated form of MMP-2 than the cell lines with a ribozyme-induced reduction in the S100A4 level. There were no visible morphological differences between actin or tubulin cytoskeletons in either of the cell lines. This shows that the S100A4-induced activation of proMMP-2 is not due to visible morphological changes in these two cytoskeletal structures. Previously, it has been reported that disruption of the microtubule network by drugs, such as colchicine, results in the induction of actin stress fibers and focal adhesions [49]. Based on this and the reported connections between the structure of the actin cytoskeleton and MMP-2 activation, one would expect that colchicine treatment of cells should result in a diminished amount of active MMP-2. In the osteosarcoma cells studied in the

present work, the effect of colchicine was only to dissolve the microtubule network but did not result in a visible change in the actin cytoskeleton. In addition to inducing dissolution of the microtubule network, colchicine treatment of the cells also resulted in the activation of MMP-2. Thus, the present work shows that the importance of cytoskeletal organization with respect to proMMP-2 activation appears to vary with cellular origin, and that the organization of the microtubules may have an effect on the status of MMP-2.

The activation of MMP-2 is a complex process that depends on both the level of MT1-MMP and TIMP-2 [6–9,50]. It is suggested that optimal activation occurs when a certain level of MT1-MMP is occupied by TIMP-2, which can link proMMP-2 to the MT1-MMP and form a ternary complex in which proMMP-2 can be activated by uncomplexed MT1-MMP [4]. In the present work, it is shown that colchicine up-regulates the steady-state mRNA levels of both MT1-MMP and TIMP-2, and that this increase results in an increased level of MT1-MMP and TIMP-2 protein in the membranes. The results of the inhibitory studies (Fig. 7D) strongly indicate that it is the MT1-MMP in the isolated membranes that is responsible for the activation of exogenous proMMP-2. This conclusion is based on the fact that the activation was inhibited by both the general MMP inhibitors EDTA and 1,10-phenanthroline, and by a high level of the specific MMP inhibitor TIMP-2, but not by general inhibitors of other classes of proteinases. The lack of inhibition by TIMP-1 further supports the above conclusion, as MT1-MMP is the only MMP that is not inhibited by TIMP-1 [51]. As the isolated membranes from the colchicine-treated cells were more effective in the activation of exogenously added proMMP-2, it must be assumed that it is the colchicine-induced alterations of MT1-MMP and TIMP-2 levels that also causes the colchicine-induced activation of endogenous proMMP-2. Consequently, as the colchicine-induced activation of proMMP-2 at cellular confluence is SN50 sensitive and hence a result of an activation of the NF-κB pathway, the colchicine-induced synthesis of MT1-MMP and TIMP-2 is also likely to be due to the activation of the NF-κB pathway.

Previously, we have shown that S100A4 induces activation of proMMP-2 in confluent and subconfluent cultures of osteosarcoma cells [16]. However, the mechanism that results in activation appears to be cell density dependent. In subconfluent cells, the S100A4-induced activation of proMMP-2 is SN50 sensitive [16]. However, at confluence the S100A4-induced activation of proMMP-2 is SN50 insensitive and hence, S100A4 acts through an NF-κB-independent pathway. Despite equal levels of MT1-MMP mRNA in pHβ-1 cells and the cells with a reduced S100A4 level at confluence, isolated membranes from the pHβ-1 cells contained higher levels of MT1-MMP and TIMP-2 protein than the membranes from the cells with a low level of S100A4. This suggests that S100A4 acts at the translational/post-translational level at cellular confluence. In the present work, it is also shown that at cellular confluence, colchicine induces activation of proMMP-2 in both the cell lines with a high level of S100A4 (pHβ-1) as well in the cell lines with a low level of S100A4 (II-11). The colchicine-induced activation of proMMP-2 in confluent cells is SN50 sensitive and hence dependent on the activation of the NF-κB pathway. This suggests that the MT1-MMP induced by S100A4 through a translational/post-translational pathway is blocked by colchicine, and that in the presence of colchicine, the regulation of MT1-MMP and the activation of proMMP-2 is entirely regulated at the transcriptional level. Previously, it has been suggested that some of the cytosolic pool of the NF-κB/IκB complex is bound to the tubulin cytoskeleton, and that tubulin-disrupting agents, such as colchicine, can activate and release NF-κB from the cytoskeleton [17–19]. Our results suggest that colchicine in addition to blocking the S100A4-induced translational/post-translational pathway also induces a release and activation of NF-κB from the microtubules that results in an activation of proMMP-2 that exceeds the SN50-independent S100A4-induced proMMP-2 activation.

In untreated II-11 cells, the synthesis of MMP-2 protein per cell has been shown to be independent of cell density, while for pHβ-1 cells it is cell density dependent [16]. At high cell density, the synthesis of MMP-2 protein was the same for the two cell lines, while at low cell density, pHβ-1 cells produced approximately twice as much MMP-2 as the II-11 cells [16]. This corresponded to the relative MMP-2 mRNA levels in the two cell lines [15]. The S100A4-induced increase in MMP-2 was inhibited by SN50 at subconfluence, which indicated that S100A4 acted through the NF-κB signaling pathway. In a recent paper [16], we found four putative NF-κB binding elements in the previously published promoter region of MMP-2 [52] by using MatInspector V2.2. These consensus p65 NF-κB binding sites were: TGGAGTTCC #4 at nt −1189 to −1181, GGGACCTTCC #1 at nt −528 to −519, GGGGAATTCC #3 at nt −1028 to −1020, and GGGTGCTTCC #2 at −930 to −921. The putative NF-κB site #3 is equal to the NF-κB site #2 in the murine Toll-like Receptor 2 (TLR2) promoter, and was shown to bind NF-κB p50/p65 [53]. This suggests that the expression of MMP-2 can be regulated by the NF-κB pathway. On the other hand, at cellular confluence the MMP-2 synthesis was not affected by SN50, suggesting an NF-κB-independent pathway. In the present work, it is also shown that colchicine has no effect on the synthesis of MMP-2 mRNA and protein at cellular confluence. In spite of this, the synthesis of MMP-2 is sensitive to SN50 in the presence of colchicine, suggesting that the synthesis is now regulated through the NF-κB pathway. Thus, colchicine has uncoupled the S100A4-independent regulation of MMP-2 and changed the regulation to a pathway that involves the activation of NF-κB.

Independent of S100A4 level, colchicine treatment changed the cell lines to a phenotype with increased

capacity to degrade extracellular matrix components. Similar results have been obtained with other cell lines treated with colchicine. It has been reported that breast cancer-mediated bone resorption *in vitro* is positively correlated with release of hydrolytic enzymes by the tumor cells, and release of these enzymes is enhanced by disassembly of the microtubules [54]. In fibroblasts and monocytes, it has been reported that colchicine alters the level of collagenase, gelatinase and ''stromelysin'' [23–25]. However, it has also been reported that fibroblasts from some patients with junctional epidermolysis bullosa do not respond to colchicine treatment by an increased collagenase synthesis [55,56]. We have previously observed similar results with skin fibroblasts from patients with various forms of skin diseases and from healthy individuals.[2] The rationale for using colchicine therapeutically varies with the disease, and in cases of dermal fibrotic diseases, such as scleroderma, which is associated with reduced turnover of the fibrotic extracellular matrix, a decreased collagen synthesis along with an increase in proteolytic enzymes, such as interstitial collagenase, appears to be beneficial [26,57]. However, as an anti-metastatic drug, one must expect the proteolytic profile to be changed to a phenotype with reduced matrix degrading properties, or that the motility of the cells is reduced. That cell lines from various origins, as well as similar cell lines from different individuals, respond differently to colchicine may be one of the explanations for the controversial results obtained in clinical trials with colchicine. Knowledge about the *in vitro* effect of a potential drug on the enzymatic profile in various cell lines may be one of the tests used to find compounds that might be of clinical interest and a guide to predict its success.

Acknowledgments

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