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Fibroblast heterogeneity in collagenolytic response to colchicine

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TIMPs, tissue inhibitors of MMPs
SBTI, soybean trypsin inhibitor
CLCS, collagenase colchicine sensitivity

ABSTRACT

Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) are important in various physiological and pathological conditions, including those that involve homeostasis of collagen. Drug induced regulation of MMP-1, other MMPs and TIMPs is critical in treatment of various diseases, e.g. the use of the plant alkaloid, colchicine. One possible factor that might explain the failure in colchicine-treatment of some patients is interindividual variability on the cellular level. To investigate the possible individual heterogeneity in response to colchicine, we studied the effect of colchicine-induced synthesis of collagenase from 32 different human skin fibroblast strains derived from both healthy individuals as well as individuals with different skin diseases. We showed that colchicine induced an increased synthesis of collagenase in 22 of 32 cases. This heterogeneity occurred in fibroblasts from healthy as well as diseased individuals. To determine if colchicine also affected the fibroblast synthesis of gelatinase, stromelysin and tissue inhibitors of MMPs, we investigated several individuals from a single family. The results showed that both colchicine responsive and non-responsive fibroblasts with respect to collagenase synthesis responded to colchicine by an increased stromelysin synthesis, while the synthesis of gelatinase and TIMP-1 were unaffected. As a whole, our results indicate that individual heterogeneity in collagenase response to colchicine treatment may partly explain some of the controversial results obtained with colchicine as a drug.

1. Introduction

The matrix metalloproteinases (MMPs) constitute a family of at least 25 different mammalian zinc and calcium-dependent enzymes, of which skin fibroblasts can express several types [1–5]. The enzyme family includes collagenases (MMP-1/-8/-13), gelatinases (MMP-2/-9), stromelysins (MMP-3/-10/-11), matrilysins (MMP-7/-26) as well as membrane-type MMPs

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(MT-MMPs) and other MMPs [4,5]. The activity of MMPs is regulated at the transcriptional, translational and post-translational levels. Most of the MMPs are synthesized in their latent pro-form, and must be converted to their active forms in the extracellular space. During activation, either parts or the entire N-terminal pro-domain are removed. This process can be performed by various agents in vitro, including *p*-aminophenylmercuric acetate (APMA), SDS, urea, chaotropic agents, heat treatment and by proteinases [6]. Once activated, the activity of MMPs can be regulated by endogenous inhibitors such as α2-macroglobulin and tissue inhibitors of MMPs (TIMPs) [7].

Together, the MMPs are able to degrade most extracellular matrix proteins, as well as regulating the activity of serine proteinases, growth factors, cytokines, chemokines and cell receptors [8]. Thus, MMPs have complicated biological functions playing a role in modulating normal cellular behaviour, cell–cell communication as well as in pathological conditions [8]. Among the naturally occurring processes that involve MMPs are wound healing and tissue remodelling, development, growth and maintenance, and examples of pathological conditions where MMPs and tissue breakdown is involved include various types of arthritis, skin diseases, oral diseases, multiple sclerosis, diabetes mellitus, cardiovascular diseases, tumor growth and angiogenesis, invasion and metastasis of tumor cells [1–5,8].

Colchicine is an alkaloid that disrupts the microtubular organisation, arrests cells in mitosis and affects other microtuble-dependent functions [9]. Due to its anti-fibrotic, anti-mitotic, anti-inflammatory and anti-metastatic activities, colchicine has been therapeutically used in the treatment of various diseases, such as gout, cirrhosis, sclerosis, aquired epidermolysis bullosa, Behcet's syndrome, Mediterranean fever and Sweets syndrome as well as in various cancers [10–20]. It has been shown that colchicine alters the expression of various MMPs (among these MMP-1), TIMPs, collagen I and other proteins in a variety of cells and tissues [21–27]. In diseases with a deposition of collagen, treatment with this kind of agents that reduce the synthesis of collagen and/or increases the synthesis of collagenases appears to be beneficial. However, in many cases the therapeutic effect of colchicine has been questioned, and sometimes treatment has resulted in severe side effects [10].

The rationale for therapeutic use of colchicine varies with the type of disease, and hence the lack of effect may be manifold. In cases where the aim is to alter the collagen homeostasis, one might expect the lack of clinical responsiveness to colchicine in some patients to be due to interindividual heterogeneity in the cellular expression of collagenases in response to colchicine. One of us (TGD) has during the last 40 years studied the genetics of various skin diseases, especially a family of blistering disorders called epidermolysis bullosa [28,29], and has therefore during the years established skin fibroblast cultures from many individuals with skin diseases as well as from healthy individuals. In the present work, we have used this material to determine if there is an interindividual heterogeneity in skin fibroblasts response to colchicine treatment with respect to the synthesis of collagenase.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA), colchicine, trypsin, soybean trypsin inhibitor (SBTI), calf thymus DNA, 3,5-diaminobenzoic acid, azocasein, casein, secondary antibodies, gelatin (Bloom 300), acid-soluble calf skin and rattail collagens were from Sigma. Culture media and fetal calf serum were from Flow Laboratories and GIBCO. Quickscint 294 was from Zinsser Analytic Ltd. Tritiated sodium borohydride and ^{14}C-formaldehyde were from Amersham. The polyvinylidene difluoride membranes used for western blotting were from Millipore, the CDP-star chemiluminiscence substrate and AP protein marker detection pack for Western blotting were from New England Biolabs. 2-Methoxy-2,4-diphenyl-3(2H)-furanone (MDPF) was from Fluka. SDS-PAGE molecular weight standards were from BioRad. Human recombinant TIMP-1 and proMMP-2 was from Oncogene and Chemicon, respectively.

2.2. Subjects

Thirty-two fibroblast cell lines were investigated, and these were obtained from 13 healthy persons, 11 patients with junctional epidermolysis bullosa (JEB: eight Gravis-Herlitz and three inversa cases), two with recessive pseudojunctional epidermolysis bullosa (REBPJ; now renamed to epidermolysis bullosa simplex with muscular dystrophy, EBS-MD [30], six with Ehlers-Danlos Syndrome (four with type III, one of type II and one of type IV). The two EBS-MD patients belong to a two generation family (NEB 1) with nine children of which eight were available for this study, and NEB1-9 and NEB1-11 are the EBS-MD children [30,31]. Three of the JEB Herlitz patients were siblings and one of the healthy controls their mother (Family EB 107).

2.3. Fibroblast cultures

Skin fibroblast cultures were established from biopsies taken from medial or lateral aspect of upper arms, as the biopsy site did not affect the collagenase production [32]. Cells from the established skin fibroblast cultures (passages 4–10) were subcultivated in disposable plastic culture dishes or flasks in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 20 mM HEPES buffer (pH 7.2–7.4), 100 IU/ml penicillin, 100 μg/ml streptomycin, nonessential amino acids (100 times dilution) and 2.0 mM L-glutamine at 37 °C in a humidified 5% CO_2 atmosphere.

To determine the effect of colchicine on the matrix metalloproteinase expression, approximately 1.5×10^5 cells were seeded per cluster 6 well to obtain optimal conditions [26]. After the cell layers were washed three times with Hank's balanced salt solution, the cultures were maintained for 48 h in serum-free DME medium containing varying concentrations of colchicine. Before freezing, half of the harvested media was made 10 mM with $CaCl_2$ and 100 mM with Hepes buffer (pH 7.5) while the other half was made 0.2% BSA/100 mM Hepes (pH 7.5)/10 mM $CaCl_2$. The former solution was used in the determination of collagenase activities, gelatin zymography and real-time reverse gelatin zymography, while

the latter solution was used in ELISA assays, Western blots and to determine the gelatinase and caseinase activities. To control and correct for minor variation in cell content within an experiment, the cell layer from each well was trypsinized and the DNA content was determined. This was performed by the previously described fluorescence method using 3,5-diaminobenzoic acid [33], using calf thymus DNA as a standard.

2.4. Immunoreactive MMPs and TIMPs

To determine the amount of immunoreactive collagenase protein (MMP-1), the previously reported enzyme-linked immunosorbent assay (ELISA) was used [32,34]. Human skin collagenase and polyclonal antibodies against human skin collagenase were kind gifts from Dr. E.A. Bauer, Stanford University.

In Western blots, cell conditioned media were electrophoresed on SDS-polyacrylamide gel (4%, w/v in stacking gel and 10%, w/v in separating gel) and transferred to a polyvinylidene difluoride membrane according to the manufature's manual. After blockage of non-specific binding sites with non-fat milk (5% solution), blots were incubated for 1 h at room temperature with primary mouse monoclonal antibodies either against MMP-3 or TIMP-1. After washing, the blots were incubated for 1 h 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.

2.5. MMP activity assays

The three MMPs (MMP-1, MMP-2 and MMP-3) are secreted into the culture medium in a latent form. They were converted to active forms by limited proteolysis using trypsin [35–39]. Briefly, aliquots of cell conditioned culture medium were mixed with serum-free DME medium (containing either 0.2% BSA/0.1 M Hepes (pH 7.5)/10 mM $CaCl_2$ (gelatinase assays) or 0.1 M Hepes (pH 7.5)/10 mM $CaCl_2$ (collagenase and stromelysin assays)) to which trypsin (0.2 mg/ml; final concentration) was added. To obtain maximal activation of the latent enzymes, these mixtures were incubated at 35 °C with aliquots withdrawn at several fixed timepoints, and the activation was terminated by inactivating trypsin with SBTI (2.2 mg/ml; final concentration). For gelatinase, the trypsin-induced activation was followed from 0–3 h, for collagenase 0–20 min and for stromelysin 0–40 min. By measuring the various enzyme activities in culture medium that has been treated with trypsin as described above, activation curves appear as described in detail previously [36,37]. The optimal activities from the curves of colchicine-treated and -untreated cells are here compared. To determine whether any of the enzymes were produced in an active form, enzyme activity was also measured on conditioned medium without trypsin activation. Collagenase activity was determined against reconstituted ^{14}C-labeled rattail collagen fibrils as a substrate [37]. The substrate used in the gelatinase assays was denatured ^{3}H-labeled calf skin collagen as described previously [36]. As stromelysin has a high activity against casein [40], azocasein was used as a substrate. The product was separated from the undegraded substrate by the method of May and Elliott [41] and the difference at A_{440nm} was used as a measure for enzyme activity [38]. All assays were performed for approximately 24 h in 0.1 M Hepes buffer pH 7.5 at 35 °C as described earlier [36–38].

2.6. Gelatin and casein zymography

SDS-substrate PAGE was performed as described previously [42], with gels (7.5 cm × 8.5 cm × 0.75 mm) containing 0.1% (w/v) gelatin or casein in the separating gel, which was 10% in polyacrylamide. In casein zymography, a pre-run of the gel was performed for approximately 2 h at a constant current of 14 mA per gel, at 4 °C prior to loading the samples onto the gel [43]. 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 homodimer (225 kDa) forms of proMMP-9 from serum-free culture medium of THP-1 cells [44,45], and 72 kDa proMMP-2 from serum free culture medium of human skin fibroblasts [46]. We also used purified 72 kDa proMMP-2 or 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/gel at 4 °C. 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 h at 37 °C. 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 and caseinolytic activity was evident as transparent zones in the blue gels. The area of the cleared zones was analysed with the GelBase/GelBlot™ Pro computer program from Ultra Violet Products, Cambridge, UK.

2.7. MMP inhibitory assays

2.7.1. Inhibitory activity in solution

The presence of MMP inhibitors in unactivated culture medium from cell strains untreated or treated with colchicine were determined as described previously [38]. Briefly, 25 μl of trypsin-activated cell conditioned medium was mixed with 0–75 μl of unactivated conditioned culture medium or unconditioned culture medium, and the collagenase inhibitory activity was determined as described previously [38].

2.7.2. Reverse gelatin zymography

Gelatin (300 bloom) was labeled with the fluorescent dye 2-methoxy-2,4-diphenyl-3(2H)-furanone to give MDPF-gelatin as described previously [42,47]. Metalloproteinase inhibitory activity was assayed by electrophoresis in polycacrylamide gels containing the fluorescent MDPF-gelatin as matrix metalloproteinase substrate and PMA-stimulated THP-1 monocyte culture conditioned media as a source of MMP. The main MMPs in this media is the monomeric form of MMP-9, and in addition it contains TIMP-1 [44,45]. Briefly, gels

(7.5 cm × 8.5 cm × 0.75 mm) containing 0.1% (w/v) MDPF-gelatin in the separating gel, was 13% in polyacrylamide and 2% (v/v) THP-1 culture-conditioned media. Prior to loading the samples onto the gel, a pre-run of the gel was performed for approximately 2 h at a constant current of 14 mA per gel, at 4 °C. 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 [48]. The presence of inhibitors appears at light fluorescence bands against a darker background due to a reduced degradation of the labelled gelatin.

2.8. Statistical analysis

To determine the effects of colchicine on the production of the different matrix metalloproteinases, data were analysed on a personal computer using the two-sample analysis procedure (*t*-statistic) of the Statgraphics program (Statistical Graphics Corporation) and One-Way ANOVA post hoc testing (SPSS Inc.).

3. Results

3.1. The effect of colchicine on collagenase synthesis

3.1.1. Immunoreactive collagenase in fibroblast conditioned media

In order to determine whether there is heterogeneity in collagenase (MMP-1) synthesis in skin fibroblasts from different individuals in response to colchicine treatment, 32 fibroblast cell lines were investigated. Cell conditioned media from colchicine-treated and -untreated cells were analysed for immunoreactive collagenase by ELISA (see Section 2). The different cell lines responses to colchicine are shown in Fig. 1. In 10 of the 32 cell lines, the synthesis of collagenase after colchicine-treatment did not differ significantly from the untreated controls, while the collagenase level was significantly increased for the other cell lines. Cell lines that showed a statistically significant increase in collagenase synthesis after colchicine treatment were regarded as positive, a trait we have designated CLCS(+), while non-responsive cells were designated CLCS(−). As shown in Table 1, responders and non-responders were found both in the panel of individuals with no known skin disease as well as in the panel of patients with skin diseases. However, as we have chosen to include some relatives in this study, the figure for unrelated individuals are 15 responsive and nine non-responsive. This suggests that skin fibroblasts response to colchicine with respect to collagenase synthesis is heterogenous.

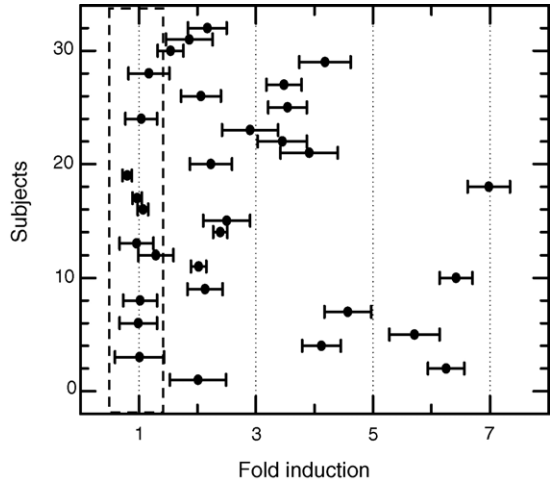

Fig. 1 – A typical scatter plot of the 32 cell lines showing the effect of colchicine on the synthesis of immunoreactive collagenase. Each cell line was treated with 0, 0.1 and 1.0 μM of colchicine. To compare the different cell lines response to colchicine, values was normalized to the corresponding cell culture not treated with colchicine. The figure shows the effect (fold induction) as mean ± S.D. for one concentration of colchicine for each cell line from one representative experiment with repeated determinations of collagenase. The cell lines within the stippled box did not significantly alter their level of collagenase in response to colchicine, and that was the case in all the independent cell experiments performed. The cell lines that responded to colchicine by a significant ($P < 0.05$) increase in collagenase synthesis did so in all independent cell experiments, but the induction could vary between 2- and 6-fold for a single cell line. The various independent cell experiments were all analysed by both two-sample analysis (*t*-statistics) and One-Way ANOVA (post hoc tests).

Table 1 – Frequency of the clcs trait in human skin fibroblast cultures[a]

Subjects	Individuals tested		
	N[b]	CLCS(+)	CLCS(−)
Healthy	13	10	3
JEB	11	7	4
EBS-MD	2	0	2
EDS	6	5	1
Total	32	22	10

[a] The effect of colchicine on the synthesis of immunoreactive collagenase from fibroblasts were determined by ELISA (see Section 2) in three to four independent cell experiments, where each cell line were treated with 0, 0.1 and 1.0 μM colchicine. Cells were regarded as CLCS(+) if the colchicine treated cell lines showed a statistically significant ($P < 0.05$) increase in collagenase synthesis compared to controls as described in Fig. 1.

[b] Number of individuals tested.

Table 2 – Effect of colchicine on collagenase activity and immunoreactive protein in fibroblast cultures from the NEB1 family

Cell line	[Colch] (μM)	Immunoreactive collagenase		Collagenase activity	
		Rel ± S.D.	P	Rel ± S.D.	P
15[a]	0	1.00 ± 0.32	–	1.00 ± 0.06	–
	0.1	2.01 ± 0.48	*	1.09 ± 0.01	NS
	1.0	3.99 ± 1.40	*	1.57 ± 0.31	*
16[a]	0	1.00 ± 0.11	–	1.00 ± 0.45	–
	0.1	1.28 ± 0.14	*	1.56 ± 0.17	*
	1.0	5.56 ± 0.94	*	2.17 ± 0.58	*
1	0	1.00 ± 0.34	–	1.00 ± 0.36	–
	0.1	–	–	0.93 ± 0.17	NS
	1.0	1.04 ± 0.40	NS	1.17 ± 0.18	NS
4	0	1.00 ± 0.11	–	1.00 ± 0.33	–
	0.1	2.41 ± 0.32	*	1.06 ± 0.05	NS
	1.0	3.09 ± 0.91	*	1.76 ± 0.13	*
5	0	1.00 ± 0.16	–	1.00 ± 0.05	–
	0.1	1.75 ± 0.51	*	1.62 ± 0.27	*
	1.0	3.49 ± 1.60	*	1.94 ± 0.33	*
9[b]	0	1.00 ± 0.24	–	1.00 ± 0.36	–
	0.1	0.99 ± 0.32	NS	1.30 ± 0.10	NS
	1.0	0.93 ± 0.28	NS	1.14 ± 0.03	NS
10	0	1.00 ± 0.24	–	1.00 ± 0.15	–
	0.1	4.45 ± 0.71	*	1.75 ± 0.65	*
	1.0	7.34 ± 3.00	*	2.27 ± 0.76	*
11[b]	0	1.00 ± 0.20	–	1.00 ± 0.12	–
	0.1	1.08 ± 0.27	NS	1.08 ± 0.17	NS
	1.0	0.98 ± 0.24	NS	0.92 ± 0.16	NS
13	0	1.00 ± 0.20	–	1.00 ± 0.20	–
	0.1	–	–	1.79 ± 0.29	*
	1.0	1.83 ± 0.42	*	1.78 ± 0.31	*
14	0	1.00 ± 0.20	–	1.00 ± 0.04	–
	0.1	2.54 ± 0.58	*	1.70 ± 0.26	*
	1.0	6.09 ± 1.09	*	1.97 ± 0.35	*

The results presented are from pooled experiments performed at different times. To be able to compare the different experiments, values were normalized to the cell-cultures not treated with colchicine. The amount of immunoreactive collagenase and collagenase activity was determined as described in Section 2. In each cell line, the amount of collagenase has been determined from 3–6 independent cell culture experiments, where *indicates $P < 0.05$ and NS, non-significant compared to untreated controls, using the student t-test two-sample analysis.
[a] Cell-line 15 and 16 are from the two healthy parents.
[b] EBS-MD patients.

3.1.2. *Collagenase activity versus immunoreactive collagenase in fibroblast conditioned media*

It is of importance to determine to what extent the cell lines synthesis of immunoreactive collagenase in response to colchicine-treatment is reflected in the biological activity of the synthesised enzyme. For that purpose, we decided to investigate an entire family (NEB1) where the immunological collagenase data revealed seven responders and three non-responders (Table 2). The family-members investigated were two siblings with the skin blistering disorder EBS-MD [30,31], six healthy siblings and the two healthy parents. No collagenase activity was detected in culture media from these individuals, with or without colchicine-treatment. This showed that the cells either did not produce active collagenase, i.e. the collagenase is secreted as a proenzyme, or that the conditioned culture media contain enough MMP-inhibitors to prevent activity. As limited trypsin proteolysis of the harvested culture media resulted in collagen degradation in all the fibroblast media tested, the collagenase appeared to be synthesised mainly as a proenzyme. As shown in Table 2, the fibroblasts synthesis of immunoreactive collagenase in response to colchicine-treatment was reflected in the collagenase activity (after limited trypsin proteolysis), although the increase in collagenase activity in responders was smaller than the corresponding increase in collagenase immunoreactive protein. Both parents were CLCS(+) and the two EBS-MD children and a healthy sibling were CLCS(−). This suggests that the CLCS(−) trait is recessively inherited in this family.

3.2. *The effect of colchicine on the synthesis of tissue inhibitors of MMPs (TIMPs)*

As the colchicine-induced increase in collagenase activity was lower than the increase in immunoreactive collagenase, it was

of interest to determine to what extent colchicine could alter the synthesis of MMP inhibitors. For that purpose, we have chosen to investigate five responders (NEB1–5, 1–10, 1–13, 1–15, 1–16) and two non-responders (NEB1–1, 1–11) from the NEB1 family using three different methods.

In our first method we determined the inhibitory activity in the culture media directly on the collagenases ability to degrade native ^{14}C-labelled collagen gels (see Section 2). No difference in the inhibitory activity of colchicine-treated and -untreated cells was observed (data not shown), indicating that colchicine did not have any effect on the synthesis of MMP inhibitors released into the cell culture medium. To verify these observations and to see if the fibroblast synthesized one or several types of MMP inhibitors, we investigated the cell conditioned medium from colchicine-treated and -untreated fibroblasts on real-time reverse gelatin zymography. Only a single band with the same M_r as TIMP-1 (28 kDa) appeared, and the intensity of this band did not vary with the concentration of colchicine. A typical reverse zymography is shown in Fig. 2(a). Western blots of cell-conditioned media verified that the 28 kDa band seen in reverse zymography is TIMP-1 (Fig. 2(b)).

3.3. *The effect of colchicine on gelatinase and stromelysin synthesis*

Other MMPs are also of importance for the homeostasis of interstitial collagen. Like MMP-1, gelatinase A (MMP-2) cleaves the triple helical region of native collagen, while stromelysin 1 (MMP-3) can cleave native collagen in the N- and C-terminal globular regions and both gelatinases, MMP-2 and MMP-9 (gelatinase B) are involved in the degradation of the denaturated collagen, i.e. gelatin [49,50]. Therefore, it was of interest to investigate to what extent the cell lines changed the synthesis of these enzymes in response to colchicine-treatment. For this purpose, we used both CLCS(+) and CLCS(−) cell lines from the NEB1 family. As for the collagenase, no stromelysin or gelatinase activity could be detected in conditioned media from colchicine-treated or -untreated cells, showing that no active forms of these MMPs were produced. However, limited trypsin proteolysis of the harvested culture media resulted in both gelatin and azocasein degradation in all the fibroblast media tested. As shown in Table 3, colchicine treatment resulted in a 2–3 fold increase in stromelysin activity (azocaseinase) from all five cell lines. Western blots showed that the changes in caseinolytic activity reflected changes in MMP-3 (Fig. 3(a)).

However, the colchicine-treatment resulted only in minor alterations in the gelatinase activity in both CLCS(+) and CLCS(−) cell lines (Table 3). Gelatin zymography was performed on conditioned media from CLCS(+) cells (NEB1–4, 1–5, 1–10, 1–13, 1–15, 1–16) and CLCS(−) cells (NEB1–1, 1–11), and a typical zymography is shown in Fig. 3(b). In all cases a strong proMMP-2 band at 72 kDa, but no active forms (66/62 kDa) appeared. The various cell lines showed a faint band at 92 kDa, i.e. a band that corresponding to proMMP-9. The band varied in intensity between the different cell lines, while no bands corresponding to active forms of MMP-9 were observed. This shows that the main contribution to the gelatinase activity after limited trypsin activation can be attributed to MMP-2. Furthermore, the lack of activity in un-activated conditioned media is due to the lack of active forms of the gelatinases. Colchicine-treatment did not result in significant changes in the amount of pro- or active forms of MMP-2 or MMP-9 in any of the cell lines investigated.

Noticeable are also that bands corresponding to the position of MMP-1 appeared in some gelatin zymograms, and these bands were stronger in the colchicine-treated CLCS(+) cells (Fig. 3(b)). However, the intensity of these bands

Table 3 – Effect of colchicine on stromelysin (caseinase) and gelatinase activity in conditioned media from NEB 1 fibroblasts

Cell Line	CLCS[a]	[Colch] (μM)	Stromelysin (caseinase)			Gelatinase		
			Activity ± S.D.	Rel.	P	Activity ± S.D.	Rel	P
16	+	0	13.0 ± 5.0	1.00	–	5159 ± 789	1.00	–
		0.1	30.3 ± 9.2	2.33	*	6209 ± 389	1.20	*
		1.0	32.1 ± 1.2	2.47	*	6322 ± 299	1.23	*
1	−	0	53 ± 8	1.00	–	6168 ± 239	1.00	–
		0.1	127 ± 16	2.38	*	6127 ± 148	0.99	NS
		1.0	186 ± 6	3.51	*	6994 ± 553	1.13	*
10	+	0	49 ± 18	1.00	–	4701 ± 450	1.00	–
		0.1	112 ± 7	2.27	*	5422 ± 249	1.15	*
		1.0	145 ± 5	2.94	*	4719 ± 353	1.00	NS
11	−	0	55 ± 5	1.00	–			
		0.1	131 ± 16	2.37	*	ND	ND	ND
		1.0	112 ± 7	2.03	*			
13	+	0	8.3 ± 2.9	1.00	–	2351 ± 94	1.00	–
		0.1	24.5 ± 1.0	2.95	*	2783 ± 129	1.18	*
		1.0	17.3 ± 1.0	2.08	*	2544 ± 94	1.08	*

The results from duplicate cell cultures and four activity measurements are presented as mean ± S.D. and as relative values. The amount of enzyme that causes a change of 0.001 A_{440nm}/min are defined as one unit of caseinase activity. The gelatinase activity is presented as cpm above background after approximately 20 h incubation at 35 °C. ND equals not done.

[a] Collagenase induction (+) and lack of induction (−) in the presence of colchicine (CLCS) is shown. *$P < 0.05$ and NS, not significant as described in Table 2.

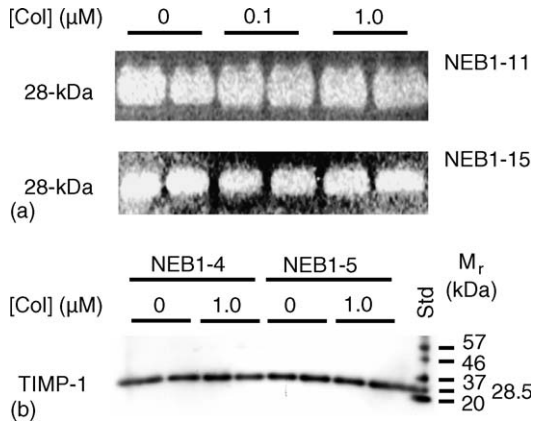

Fig. 2 – Detection of MMP inhibitors. (a) Conditioned media from fibroblast cell lines NEB1–11 and NEB1–15 treated with the indicated concentrations of colchicine were analysed by real-time reverse zymography. The zymogram was calibrated as described in methods. (b) Conditioned media from cell lines NEB1–4 and NEB1–5 treated with the indicated concentrations of colchicine were analysed by Western blotting using antibodies against TIMP-1. Shown at the right is the M_r of the standard markers.

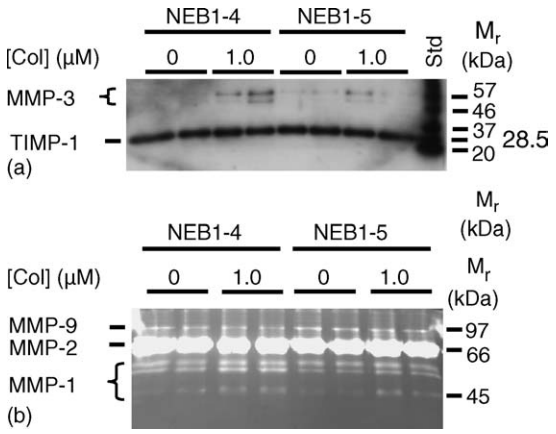

Fig. 3 – Detection of MMPs by: (a) Western blotting and (b) gelatin zymography. (a) Conditioned media from cell lines NEB1–4 and NEB1–5 treated with the indicated concentrations of colchicine were analysed by Western blotting using antibodies against MMP-3. As a control, the blot was also stained with antibodies against TIMP-1, a protein not affected by colchicine-treatment in the cell lines used in the present work. Shown at the right is the M_r of the standard markers. (b) A representative gelatin zymogram showing the effect of colchicine on the synthesis of gelatinases and collagenases from the fibroblast cell lines NEB1–4 and NEB1–5. Shown at the left side is the position of unreduced proMMP-9 (92 kDa) from THP-1 cells, recombinant proMMP-2 (72 kDa) and MMP-1 from TNFα-stimulated fibroblasts. At the right side is the M_r from a reduced standard M_r marker kit shown.

were the same in colchicine-treated and -untreated CLCS(−) cells. The bands around 55 kDa were more intense than the 72 kDa gelatinase in casein gels (data not shown).

4. Discussion

Trypsin cleaves proMMP-1 into an inactive intermediate that is autocatalytically converted to an active MMP-1 [35,51,52]. Previously it was also shown that active MMP-1 did undergo autolysis resulting in truncated forms of the enzyme where none of the forms had collagenase activity, whereas two of the truncated forms had gelatinolytic and caseinolytic activity [53]. We have recently shown that trypsin-treatment of proMMP-2 results in both activation and inactivation/degradation of the enzyme, and that the balance between these two processes could be controlled to a certain extent by various environmental factors [39]. That the colchicine-induced increase in immunoreactive collagenase was larger than the corresponding increase in collagenase activity in the present work could not be explained by a parallel increase in TIMP-1 or other MMP inhibitors. A likely explanation is that limited trypsin proteolysis of the cell-conditioned culture medium results in two different processes, the removal of the N-terminal pro-domain of the enzyme which results in the activation, and enzyme degradation. Thus, only a part of the total amount of proMMP-1 will form an active enzyme and the rest is inactive due to either a degraded enzyme or an inactive enzyme intermediate.

Previously it was shown that colchicine could stimulate human skin fibroblasts to increase the synthesis of collagenase [26]. It has also been reported that skin fibroblasts from some patients with a slightly atypical form of the bullous skin disease junctional epidermolysis bullosa (JEB) were non-responsive to colchicine with respect to collagenase synthesis [54,55]. In addition, it has been shown that colchicine only minimally stimulated collagenase mRNA in cultured neonatal foreskin fibroblasts [56]. In the present work it is shown that fibroblasts from different individuals (both healthy and diseased) can be divided into two groups: those that respond to colchicine by an increase in collagenase and those that do not change the collagenase synthesis. Altogether, there is a strong indication that there is heterogeneity in the population with respect to their skin fibroblasts ability to respond to colchicine-treatment in vitro. Other examples of similar individual heterogeneity in cellular response to a drug have been found for human gingival fibroblast in the synthesis of collagenase as response to cyclosporine [57] and in the induction of stromelysin mRNA by TNF in human synovial fibroblasts [58].

The effect of colchicine on the synthesis of collagenase from different cell types and species has to some extent been investigated. Treatment with colchicine resulted in an increased synthesis of collagenase from fibroblast and fibroblast like cell lines from different species, such as human periodontal ligament fibroblasts [59], rabbit synovial fibroblasts [60] and adherent rheumatoid synovial cells [61]. However, the effect of colchicine on collagenase synthesis from different blood cells varied. Whereas an increase in the synthesis of collagenase was found in mouse peritoneal macrophages [25], no effect was shown in human leukocytes

[62], and a decrease was shown both in guinea pig macrophages [63,64] and human peripheral blood monocytes [65]. This shows that the effect of colchicine on collagenase synthesis varies with both cell type and species, although, there are to our knowledge no other studies than the present one and that from Tipton et al. [57] that have reported on inter-individual differences in the synthesis of collagenase from a single cell line in response to a drug.

In addition to collagenases, other enzymes like gelatinases and stromelysins are involved in the degradation of interstitial collagen. The two gelatinases MMP-2 and MMP-9 are involved in the degradation of denatured collagen (gelatin) and MMP-2 can also act as a collagenase, although with a much lower specific activity than the collagenases [50]. Of the two gelatinases, skin fibroblasts produce mainly MMP-2, and the present work shows that colchicine has no effect neither on the synthesis nor on the activation of this gelatinase in human skin fibroblasts. It is of importance to emphasize that in this case we have only studied one family, so we cannot exclude that there are inter-individual differences in the synthesis of MMP-2 from skin fibroblasts in response to colchicine. Recently we showed that colchicine-stimulation of osteosarcoma cells resulted in increased activation of MMP-2, but no alteration in the total synthesis of this enzyme [47]. However, the effect of colchicine on MMP-9 synthesis appears to vary with cell type as it increased from mouse peritoneal macrophages [25], decreased from mouse primary hepatocytes [66] and no effect was shown either on a human melanoma cell line [67] or on human brain microvascular endothelial cells cocultured with glioblastoma cells [68]. In the present work, no induction of MMP-9 from fibroblasts in response to colchicine-treatment was observed. Altogether this shows that the effect of colchicine on the synthesis and activation of MMP-2, and MMP-9 varies largely with cell type, and from an in vivo perspective, one can assume that colchicine as a drug has different effects on different tissues.

Previously it was shown that colchicine increased the synthesis of azocaseinase (stromelysin) from mouse peritoneal macrophages [25]. In the present work it was shown that colchicine increased the synthesis of stromelysin in both CLCS(+) and CLCS(−) fibroblasts. Thus various cell types appear to respond to colchicine-treatment by an increased synthesis of MMP-3, and it also appears that at least in the individuals studied in the present work, the heterogeneity of the colchicine-induced induction of MMP-1 is not reflected in the synthesis of MMP-3. In the present work, neither of the fibroblasts investigated responded to colchicine by an increase in TIMP-1 synthesis, similar to what we observed previously for human osteosarcoma cells [47]. However, it has been reported that colchicine increased the synthesis of TIMP-1 in human periodontal ligament fibroblasts [59]. This shows that different cell types respond differently to colchicine with respect to TIMP-1 expression, and inter-individual differences in human skin fibroblasts cannot be excluded since only a single family was studied in the present work. However, there are so far no reports on individual heterogeneity in specific cell lines with respect to colchicine-induced TIMP-1 or MMP-3 synthesis.

In fibrotic diseases such as systemic sclerosis, a characteristic trait is thickening of the skin due to an excessive accumulation of extracellular matrix components, especially collagen, in the lesional tissue [69]. In addition to resident cells such as fibroblasts, blood cells such as monocytes/macrophages are recruited to the lesional tissue probably due to the production of monocyte chemoattracant proteins (MCPs) from fibroblasts in the lesional tissue [70–72]. Fibroblasts are also the producers of both collagen and factors that is involved in the regulation of collagen degradation such as various MMPs and TIMPs. Skin fibroblasts are therefore a very useful cell-model in the study of factors that are of importance in the homeostasis of collagen and the possible heterogeneity in the synthesis of such factors. In the degradation of interstitial collagen, MMP-1 is the main enzyme that cleaves in the triple helical region. The cleaved collagen melts and generates gelatin which can be further cleaved by other enzymes such as the gelatinases (MMP-2 and MMP-9). In addition will other proteinases such as MMP-3 cleave, both the native and denatured collagen in the N- and C-terminal globular cross-linked regions, and hence fully release degraded collagen from intact fibrils [49]. MMP-3 can also affect collagen metabolism by: (a) its ability to super-activate MMP-1, and hence increase the specific activity against native collagen [6] and (b) by the ability to degrade the small proteoglycan decorin which bind and regulate the collagen fibre formation [49]. During conditions with a defect collagen homeostasis such as in various fibrotic conditions, treatment that alters both the synthesis of collagen and collagen degrading proteinases appears to be beneficial. A typical drug that can influence these properties is colchicine, which has also been used in the treatment of fibrotic diseases such as sclerosis, although with controversial results [11,12,73–75]. Based on the results in the present paper one can assume that one of the factors that may contribute to the controversial clinical results with colchicine is that the target cells response to colchicine may vary from individual to individual. Another good example of such individual heterogeneity in drug response is the effect of cyclosporine on human gingival fibroblasts ability to synthesise collagenase, which in part has been suggested as an explanation of the fact that some but not all patients that is treated with cyclosporine develops gingival fibrosis [57]. To conclude, the development of a reliable system for in vitro testing of relevant cell lines from patients in question may be important to identify non-responders, and thereby avoiding ineffective drug treatment and possible unwanted side effects.

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