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Distinct pathways in the over-expression of matrix metalloproteinases in human fibroblasts by relaxation of mechanical tension

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Abstract

The aim of the work was to analyze, on a comparative basis, the signaling pathways operating in the regulation of a panel of matrix metalloproteinases (MMP) expressed by human dermal fibroblasts submitted to mechanical stress relaxation by cytochalasin D (CD) and in a retracting collagen gel (RCG). The mRNA steady-state level of MMPs was measured by a quantitative RT-PCR procedure using a synthetic RNA as internal standard. In monolayer, most MMPs were barely detected, except MMP-2. Disruption of the actin stress fibers by CD induced a moderate increase of MMP-2 mRNA and a much larger stimulation of MMP-3, -9, -13 and -14 mRNAs. In RCG, a significant up-regulation of these MMPs was also observed although to a lower extent than in CD-treated monolayers. Among the investigated MMPs, the MMP-8 and -11 were not reproducibly detected. MMP-2 was processed to its active form both by CD and in RCG. The CD-induced up-regulation of gene expression was largely repressed by blocking protein synthesis by cycloheximide for all the MMPs, by inhibiting the tyrosine-kinases of the src family by herbimycin A for all MMPs, except MMP-2, and by inhibiting the TPA-inducible PKC isoforms by bisindoyl maleimide for all MMPs, except MMP-14. The up-regulation induced by stress relaxation in RCG was protein synthesis-dependent for MMP-2 and MMP-13, tyrosine kinases-dependent for MMP-3 and MMP-13, as previously described for MMP-1. Inhibiting TPA-inducible PKC did not affect any MMP in RCG except MMP-13, which was strongly induced. The processing of MMP-2 was tyrosine kinases-dependent but PKC-independent. Inhibitors of the ERK1,2 and p38 MAP kinases pathways diversely affected the MMPs expression. Inhibiting the Rho-kinase activity by Y-27632 was inactive. These results point to the potent regulation operated by the status of the cytoskeleton on the cell phenotype, and to distinct regulatory pathways involved in the control of different MMPs expression.

Keywords : Matrix metalloproteinases ; Actin stress fibers ; Signaling ; RT-PCR ; Biomechanics

Abbreviations : BIM, bisindoyl maleimide ; CD, cytochalasin D ; CHX, cycloheximide ; ECM, extracellular matrix ; HbA, herbimycin A ; MMP, matrix metalloproteinase ; mssRNA, multistandard synthetic RNA ; MT-MMP, membrane type MMP ; PKC, protein kinase C ; PMSF, phenyl methyl sulfonyl fluoride ; RCG, retracting collagen gel ; TK, tyrosine kinase ; TPA, tetradecanoyl phorbol acetatel.

1. INTRODUCTION

Interactions of cells with the extracellular matrix are essential in many biological and pathological processes such as embryonic development, wound healing, fibrosis, tumor invasion and metastasis. The information contained in specific sequences within the extracellular macromolecules, and triggered by their mechanical functions, are transduced in the cells through the integrins. The intracellular domain of these transmembrane receptors is connected to a complex of structural and signaling proteins forming the focal adhesions that anchor the actin stress fibers to the cell membrane. The regulation operated by the integrins on the cell phenotype is, at least in part, conditioned by the mechanical rigidity of the ligand. The resistance offered by the support applies tension on integrins, increases the stiffness of the cytoskeleton (Wang et al., 1993), the strength of the integrin-cytoskeleton linkage (Choquet et al., 1997) and the assembly and signaling activity of focal adhesions proteins (Chrzanowska-Wodnicka and Burridge, 1996; Pelham and Wang, 1997). It results in the phenotype displayed by fibroblasts in monolayer on a rigid culture dish, with a dense network of actin stress fibers anchored to focal adhesions. Decreasing the tensional force by disruption of the cytoskeleton by cytochalasin D (CD), or by culturing fibroblasts in a floating freely retracting collagen gel (RCG), produces drastic changes, expressed by: a decrease of the multiplication rate; commitment to apoptosis; and regulated expression of specific genes

(Nusgens et al., 1984; Unemori and Werb, 1986; Mauch et al., 1988, 1989; Lambert et al., 1992, 1998; Fluck et al., 1998). Notably, a large up-regulation of MMP-1 is observed both at the protein and mRNA levels. The over-expression of MMP-1 in RCG is dictated by the mechanical status of the cells since it is largely repressed when they are maintained under tension by preventing gel retraction (Lambert et al., 1992). The signaling pathway operating in the RCG-induced MMP-1 over-expression is mediated through the $a_2\beta_1$ inte-grin (Langholz et al., 1995; Riikonen et al., 1995), and involves the activation of tyrosine kinases and the expression or activation of transacting factors (Broberg and Heino, 1996; Lambert et al., 1998). The over-expression of MMP-1 induced by CD further depends on the activity of TPA-dependent protein kinase C (PKC) isoforms while that in RCG does not (Lambert et al., 1998). MMP-13 is also over-expressed in RCG. This regulation is mediated by the integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$, involves tyrosine kinase(s) and depends on a balance between the activity of p42/44MAPK and p38MAPK (Ravanti et al., 1999). A processing of proMMP-2 to MMP-2 by CD and in RCG has also been reported (Seltzer et al., 1994; Tomasek et al., 1997). The regulation of other MMPs upon stress relaxation has received little attention.

Matrix metalloproteinases (MMPs) are a family of genetically-related zinc-dependent proteases that collectively degrade most components of the extracellular matrix (ECM) with distinct specificities. They play a major role in physiological and pathological ECM remodeling processes such as: placental implantation; morphogenesis; wound healing; periodontitis; arthritis; and tumoral progression and invasion. 'Archetypal' MMPs are secreted as latent pro-enzymes and activated extracellularly by cleavage of the prodomain. This cleavage is auto-catalytic, or mediated by different enzymes, including other MMPs and serine proteases as plasmin (Nagase and Woessner, 1999).

The aim of the present work is a comparative analysis of the signaling pathways operating in the regulation of the panel of MMPs expressed by human dermal fibroblasts in vitro under different mechanical conditions. The mRNA steady-state level of the major MMPs produced upon dissipation of mechanical tension by CD and in RCG was measured by a non-competitive quantitative RT-PCR procedure (Freeman et al., 1999) using an original multi-standard synthetic RNA (mssRNA) to monitor the efficiency of the assay.

2. RESULTS

2.1. Selection of primers for RT-PCR

The pairs of primers (see Table 1) allowing the detection by RT-PCR of human and mouse MMP-2, -3, -8, -9, -11, -13 and -14 mRNA, and of the 28S rRNA, were selected from published sequences on the basis of the following criteria: (1) when possible a single pair of primers should allow the detection of both human and mouse RNA, a condition met for all of the RNA of interest except MMP-9 that required separate sets of primers; (2) RT-PCR products should range between 150 and 250 bp in length; (3) when possible the primers should be located on different exons to allow discrimination by electrophoresis between genuine RT-PCR products and products amplified from possibly contaminating DNA; (4) a theoretical melting temperature of 72 °C calculated by using 4 °C for GC and 2 °C for AT; (5) a minimum complementarity between the forward and the reverse primers sequences.

The efficiency of the selected primer pairs for each mRNA was tested by RT-PCR using 10 ng of total RNA extracted from human or murine cells and tissues (Fig. 1). All the RT-PCR products had the expected size. The assay was also performed using 50 ng of human and mouse DNA. If detected, the reaction products had a larger size than the amplification products obtained from cellular RNA due to the presence of intronic sequences (not illustrated). The only exception was the intronless 28S rRNA. No signal was detected in control samples free of nucleic acid.

Table 1: *Sequence of forward and reverse primers used for RT-PCR amplification of the target RNA and length of the RT-PCR products*

RNA species	Forward (5'-3')	Reverse (5'-3')	Length (bp) of the RT-PCR product from:	
			Endogenous RNA	mssRNA
h,mMMP-2[a]	AGATCTTCTTCTTCAAGGACCGGTT	GGCTGGTCAGTGGCTTGGGGTA	225	271
h,mMMP-3	GATCTCTTCATTTTGGCCATCTCTTC	CTTCCAGTATTTGTCCTCTACAAAGAA	246	272
h,mMMP-8	CCAAGTGGGAACGCACTAACTTGA	TGGAGAATTGTCACCGTGATCTCTT	200	267

hMMP-9	GCGGAGATTGGGAACCAGCTGTA	GACGCGCCTGTGTACACCCACA	208	266
mMMP-9	GTTTTTGATGCTATTGCTGAGATCCA	CCCACATTTGACGTCCAGAGAAGAA	208	271
h,mMMP-11	ATTTGGTTCTTCCAAGGTGCTCAGT	CCTCGGAAGAAGTAGATCTTGTTCT	155	268
h,mMMP-13	ATGATCTTTAAAGACAGATTCTTCTGG	TGGGATAACCTTCCAGAATGTCATAA	203	270
h,mMMP-14	GGATACCCAATGCCCATTGGCCA	CCATTGGGCATCCAGAAGAGAGC	221	269
28S	GTTCACCCACTAATAGGGAACGTGA	GGATTCTGACTTAGAGGCGTTCAGT	212	269

[a]Abbreviations: MMP: matrix metalloproteinase; 28S: 28S rRNA; h: human; m: mouse.

Fig. 1. *Electrophoretic analysis of the RT-PCR products from human (h) and mouse (m) RNA, and from mssRNA. Total RNA (10 ng) prepared from human dermal fibroblasts treated with TPA (hMMP-2, -3 and -14 and 28S); human neutrophils (hMMP-8), HT1080 treated with TPA (hMMP9); human placenta (hMMP-11 and -13); 3T3 fibroblasts treated with TPA (mMMP-2, -13 and -14 and 28S), or in RCG (mMMP-3); mouse neutrophils (mMMP-8) and mouse placenta (mMMP-9 and -11); or 10^6 copies of CTR1 (1) or CTR2 (2) mssRNA were submitted to RT-PCR using the specific primers described in Table 1, and analyzed by polyacrylamide gel electrophoresis as described. No: control reaction in the absence of RNA. Number of cycles: 19 (28S rRNA); 20 (CTR1 or CTR2); 30 (MMP-2 and -14); 33 (MMP-3 and -13); and 35 (MMP-8, -9 and -11 and water).*

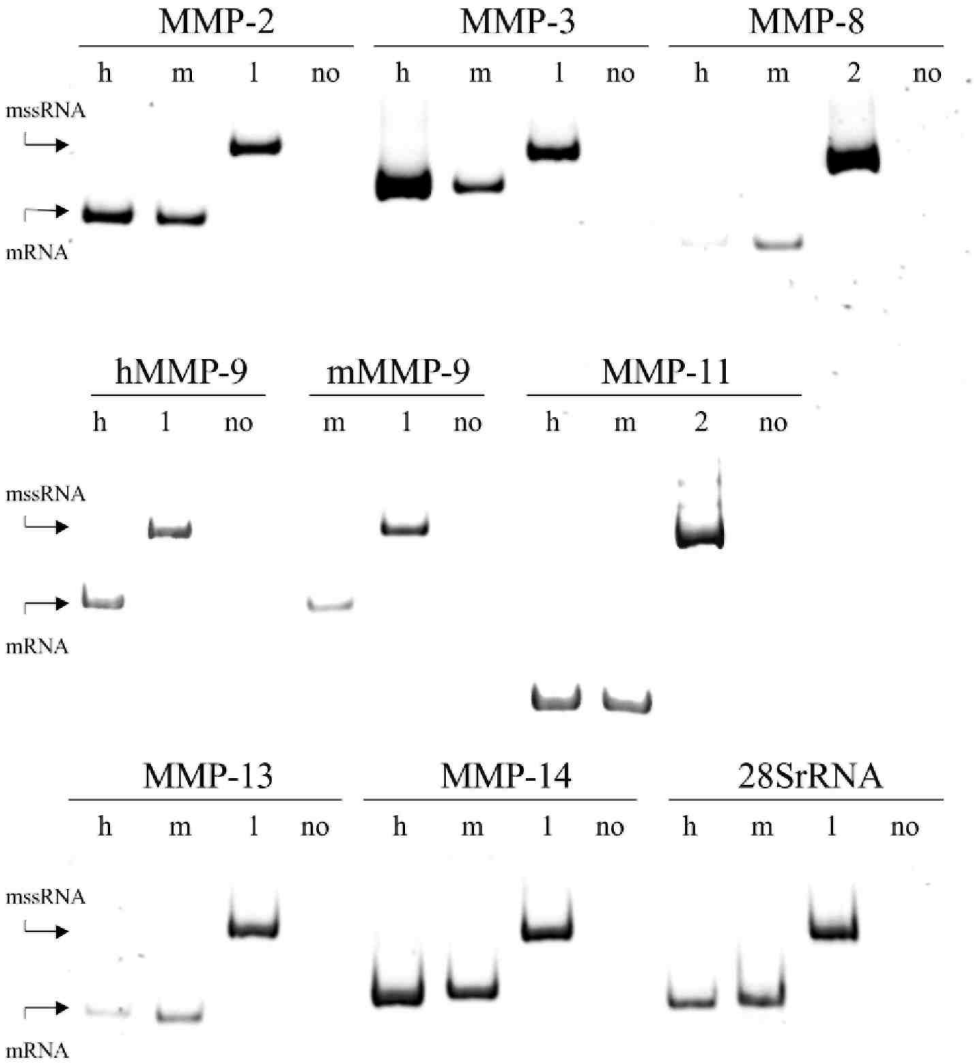
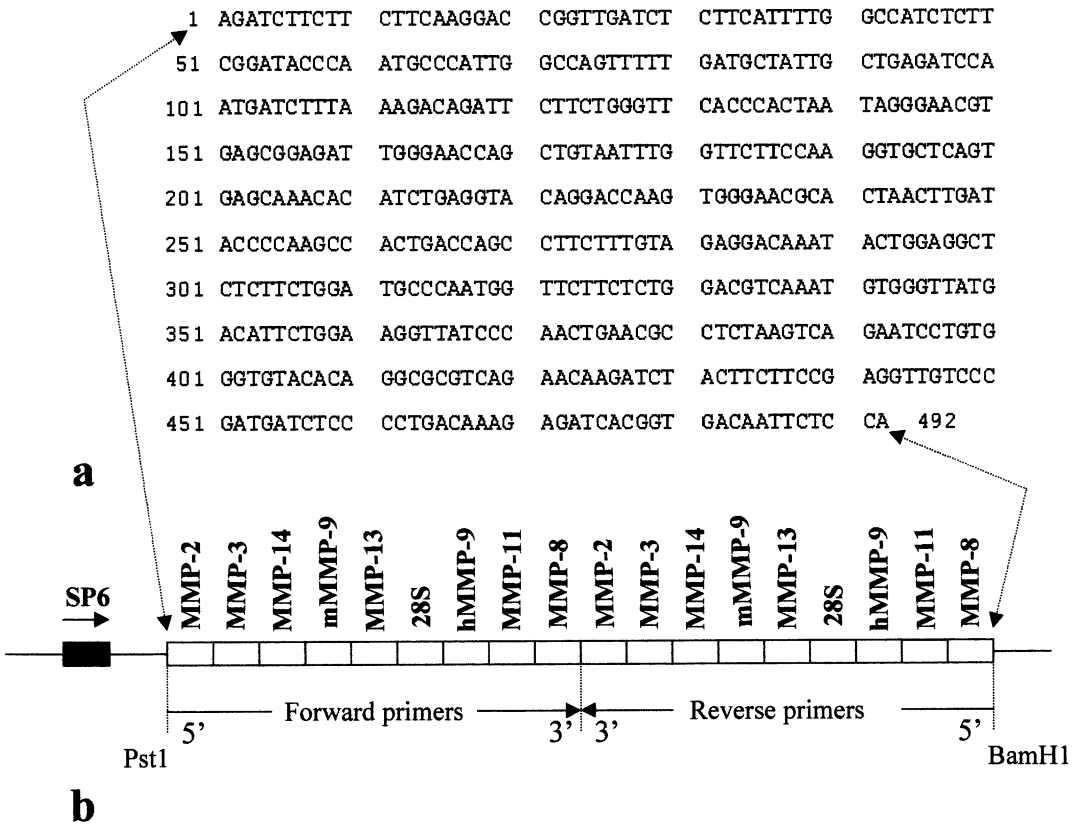


Fig. 2. *Sequence of the multiple standard DNA and map of the plasmids pCTR. (a) Expected sequence of the multiple standard synthetic DNA. The sequence of the four oligonucleotides assembled to generate this DNA corresponds to bases 1-141 and 233-375, or complementary to bases 257-116 or 492-351. (b) Arrangement of the forward and reverse primers of the target RNA (open boxes) and SP6 polymerase site (black box) in the pSPT18 plasmid (line). MMP: matrix metalloproteinase; 28S: 28S rRNA.*



2.2. Construction and use of the multi-standard RNA

Four partially overlapping 150 bases long oligonucleotides were assembled by overlap extension and subsequent amplification by PCR (Ho et al., 1989), resulting in the synthesis of a 492 bp long construct containing adequately spaced sequences of the forward primers, and complementary sequence to the reverse primers selected for the various RNAs, including the 28S rRNA (Fig. 2). The full-length product was purified and cloned in pSPT18 transcription vector (SP6/T7 transcription kit, Roche Molecular Biochemicals). Synthetic RNA was generated from the linearized template plasmid by SP6 RNA polymerase, purified and quantified. RT-PCR amplification of this multiple standard synthetic RNA (ms-sRNA) using the different pairs of primers generated amplification products larger (266-272 bp) than those obtained from the cellular RNA (155-246 bp, see Table 1 and Fig. 1), enabling their discrimination by electrophoresis. All the tested clones diverged from the expected sequence by point mutations. However, the mssRNA derived from two clones, designed CTR1 and CTR2, allowed the quantification of all the genes of interest. RT-PCR reactions using the specific primer pairs, and the mssRNA CTR1 or CTR2, yielded products of the expected size (Fig. 1).

A defined number of copies of the mssRNA CTR1 or CTR2 was added to each sample to monitor variations in efficiency of the RT-PCR reaction. The noncompetitive conditions, as defined by Freeman et al. (1999), allowed quantification of the various RNAs as illustrated for the MMP-14 mRNA in the following experiment. Increasing amounts of a cellular RNA, from 0.5 to 10 ng, and a constant number of copies of mssRNA were amplified by RT-PCR. The ratio between the intensity of the signal obtained for the cellular RNA and that for the mssRNA increased linearly with the amount of cellular RNA (Fig. 3) validating the RT-PCR quantification assay. In all assays, the actual concentration of the specific mRNA calculated as copy number equivalent to mssRNA was expressed per unit of 28S rRNA measured on the same solution of RNA.

2.3. Regulation of human fibroblasts MMPs by disruption of the cytoskeleton with CD and in RCG

The steady-state level of the mRNA coding for human MMP-2, -3, -8, -9, -11, -13 and -14 was measured by RT-PCR in a number of independent experiments in untreated and CD-treated human fibroblasts monolayers and in RCG. Two days of culture was selected on the basis of previous work showing that the expression of MMP-1 in CD-treated monolayer and in RCG was largely increased after 48 h (Lambert et al., 1992, 1998). One typical set of electrophoretic patterns of the amplification products of the mRNA, and the mssRNA for the investigated MMPs, is illustrated in Fig. 4. A substantial amount of the mRNA of MMP-2 was found in control monolayers while the mRNA of the other MMPs was barely detected. CD induced a moderate increase of the mRNA of MMP-2, and a much larger enhancement of MMP-3, -9, -13 and -14 mRNA steady-state level. In RCG, the increase of MMP-2 and MMP-14 mRNA was in the same order of magnitude as that induced by CD. The other investigated MMPs were significantly up-regulated in RCG but to a lower extent than by CD. A low expression coupled to a weak and non-reproducible induction by CD or RCG was observed for MMP-8 and -11 (not shown). These MMPs were not further investigated. As the values observed upon treatment with CD were consistently the highest, they were considered as 100% for investigating, on a comparative basis, the signaling pathways operating in the stress relaxation-mediated regulation of the MMPs.

2.4. Effect of protein synthesis inhibition

The requirement for protein synthesis in the stress relaxation-induced over-expression of MMP-2, -3, -9, -13 and -14 was investigated by treating cells with cycloheximide (CHX) at 20 μg/ml for 3 h prior to seeding in RCG or in monolayer, with or without CD. At this concentration, the drug inhibited protein synthesis by 98%, and suppressed the over-expression of MMP-1 induced by CD or in RCG (Lambert et al., 1998). In control monolayers, blocking protein synthesis did not significantly affect the basal level of MMP-3, -9 and -13 mRNA (Fig. 5) while MMP-2 was significantly lowered and MMP-14 increased. The up-regulation induced by CD was largely repressed by CHX and reduced to a level similar to that measured in monolayer treated with the inhibitor. The RCG-in-creased mRNA level was significantly reduced for MMP-2 and -13. The increase of MMP-9 induced in CHX-treated cells cultured in RCG did not reach statistical significance.

Fig. 3. *Validation of the RT-PCR. The MMP-14 mRNA was measured in increasing amounts of cellular RNA (in ng) from fibroblasts cultured in RCG (25 cycles of amplification). The same copy number of mssRNA was introduced in each sample and the ratio between the signals recorded for the cellular RNA and the mssRNA was plotted against the amount of cellular RNA.*

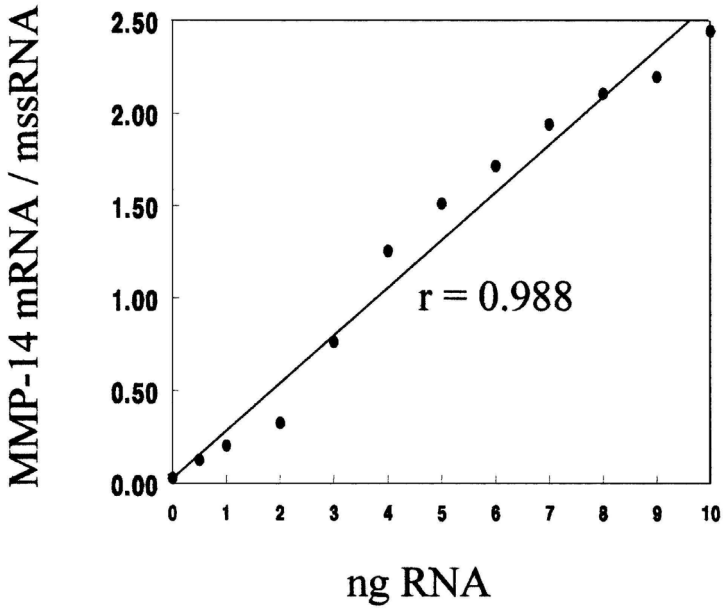


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Fig. 4. *Representative pattern of expression of the MMPs in control and CD-treated monolayers and in RCG. The expression of the various mRNAs in monolayer of human dermal fibroblasts untreated (a), or treated with CD (b), or cultured in retracted collagen gel (c), was measured by RT-PCR as described. Asterisks indicate mssRNA RT-PCR product. MMP-2, -3 and -14: 2.5 × 10^5 copies of mssRNA, 25 cycles; MMP-9: 100 copies of mssRNA, 38 cycles; MMP-13: 350 copies of mssRNA, 37 cycles; 28S rRNA: 10^6 copies of mssRNA, 19 cycles.*

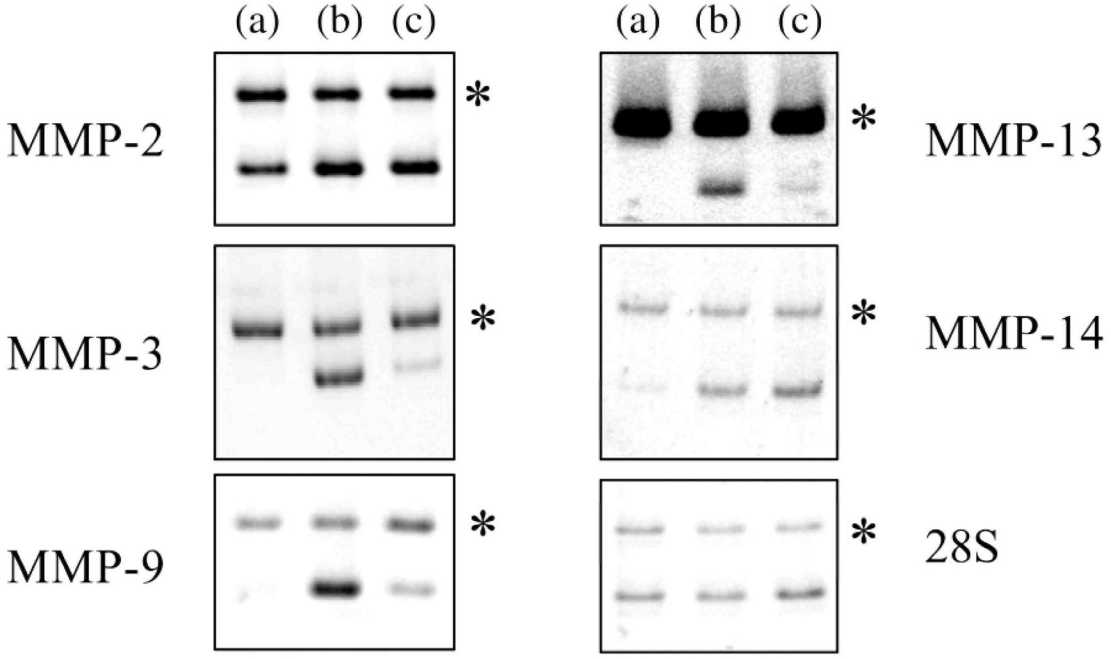

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Fig. 5. *Steady-state level of MMP-2, -3, -9, -13 and -14 mRNA in control (C) and CD-treated (CD) monolayers and in retracted collagen gel (RCG) and effect of inhibitors of protein synthesis, tyrosine kinases and PKC activity on the expression.Fibroblasts were untreated (open bars), or treated (cross-hatched bars) with cycloheximide (CHX, 20 μg/ml), herbimycin A (HbA, 260 nM), or bisindoylmaleimide (BIM, 5 μM). After 2 days of treatment, the expression of the various mRNA was measured by RT-PCR as described. The data are expressed in % of the values recorded in CD-treated monolayers as the mean + S.D. of three to 11 independent experiments. *P ≤ 0.05.*

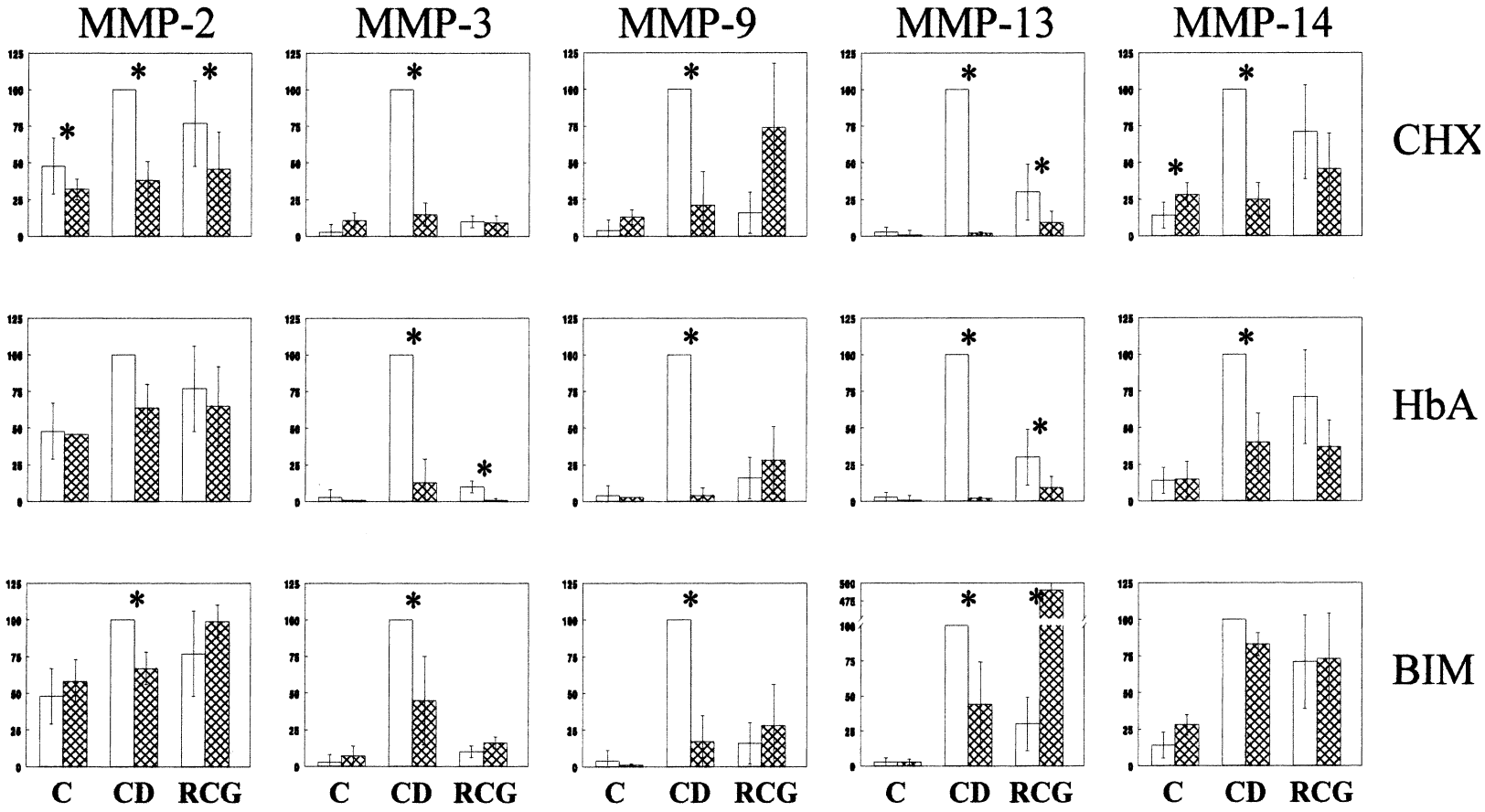
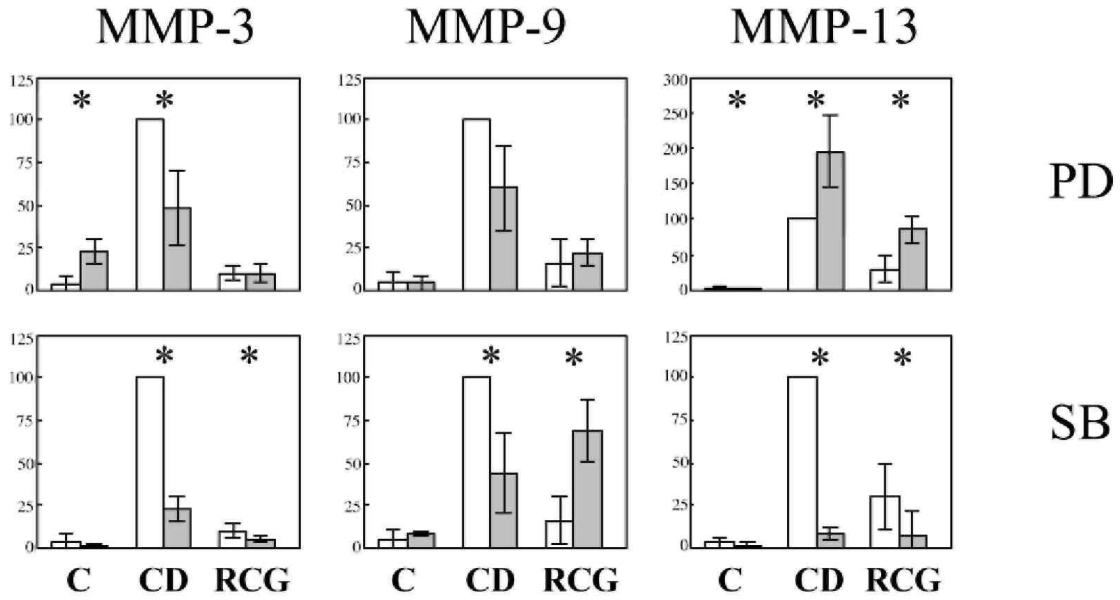


Fig. 6. *Effect of inhibition of MAPkinases on the steady-state level of MMP-3, -9 and -13 mRNA in control (C) and CD-treated (CD) monolayers and in retracted collagen gel (RCG). Fibroblasts were untreated (open bars), or treated (cross-hatched bars) with PD98059 (PD, 50 μM), or SB203580 (SB, 20 μM). After 2 days of treatment, the expression of the various mRNA was measured by RT-PCR as described. The data are expressed in % of the values recorded in CD-treated monolayers as the mean + S.D. of three independent experiments. *P ≤ 0.05.*



2.5. Role of tyrosine kinases

The involvement of protein tyrosine kinases (TK) in the CD- or RCG-induced regulation of the MMPs was investigated by using the TK inhibitor herbimycin A (HbA) at 260 nM (Fig. 5). This concentration was shown to suppress the CD- and RCG-induced expression of MMP-1 without affecting cell viability or gel contraction (Lambert et al., 1998). Fibroblasts were treated overnight with HbA, trypsinized and seeded in monolayer with and without CD, or in RCG which was cultured for 48 h in presence of HbA, before collecting the RNA. HbA did not affect the steady-state level of MMPs mRNAs in monolayer. The CD-induced up-regulation of MMP-3, -9, -13 and -14 was largely depressed by blocking tyrosine kinases, while MMP-2 was not significantly affected. The RCG-induced over-expression of MMP-3 and -13 was suppressed by HbA.

2.6. Role of PKC

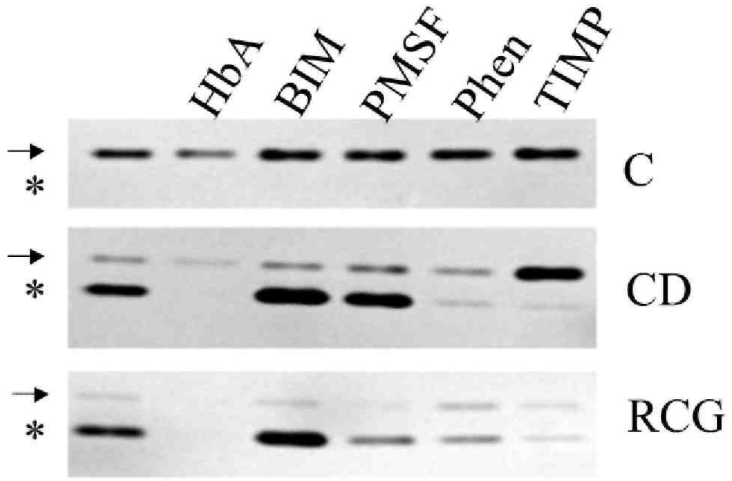
The role of the TPA-dependent PKC isoforms in the CD- or RCG-induced expression of the MMPs was assessed by using bisindoyl maleimide (BIM) at 5 μM (Fig. 5). This concentration decreased the PKC activity in TPA-treated fibroblasts by a factor of 6.0 ± 1.5, i.e. below its basal level, and suppressed the TPA-and CD-induced over-expression of MMP-1 (Lambert et al., 1998). BIM had no effect on cells in monolayer and significantly reduced the CD-induced over-expression of all the MMPs except that of MMP-14. The RCG-induced over-expression was barely affected except that of MMP-13 that was strikingly increased in RCG by the PKC inhibitor.

2.7. Role of MAP kinases and Rho-kinase

The participation of the MAP kinases ERK1,2 and p38 and Rho-kinase in the described regulations was investigated by using the inhibitors of MEK1,2 (PD98059, 50 μM), of p38 (SB203580, 20 μM) and Rho-kinase (Y-27632, 10 μM). Cells were pre-treated with the inhibitors for 3 h before adding CD or before inclusion in the RCG. The increased level of MMP-3, -9 and -13 mRNA induced by CD-treatment of monolayer was largely repressed upon inhibition of p38 MAP-kinase (Fig. 6). Similarly, this inhibitor repressed the increased level of MMP-3 and -13 in RCG but further elevates the MMP-9 mRNA. MMP-2 was moderately affected by SB203580 and MMP-14 not affected at all (not illustrated). PD98059 increased MMP-3 expression in control monolayer,

while it decreased its CD-induced over-expression (Fig. 6). In contrast it decreased MMP-13 expression in control monolayer, while it increased it in CD-treated monolayer and in RCG. The expression of the other genes was not affected by MEK1,2 inhibition. Inhibition of the Rho-kinase by Y-27632 did not affect the expression of the various MMPs (not illustrated).

Fig. 7*. MMP-2 activity and processing. Conditioned medium from fibroblasts cultured in control (C) and cytochalasin D-treated monolayers (CD), and in retracting collagen gel (RCG) were analyzed by zymography. Samples were untreated, or treated with: herbimycin A (HbA, 260 nM); bisindoyl maleimide (BIM, 5 μM); phenylmethylsulfonylfluoride (PMSF, 1 mM); 1,10 O-phenanthro-line (Phen, 1 mM); or tissue inhibitor of metalloproteinase 2 (TIMP, 100 ng/ml). The gels were analyzed using a Fluor-S Multi-Imager and the color inverted to increase the contrast. Arrow: pro-MMP-2; *: activated MMP-2.*



2.8. Activation of MMP-2

Serum-free conditioned medium of fibroblasts untreated or treated with CD, or cultured in RCG for 2 days, were analyzed for gelatinase activity by zymography. MMP-2 was present in the control monolayer in a 72-kDa form corresponding to its latent form. It was processed to the 60-kDa active form in the conditioned medium from fibroblasts treated with CD or cultured in RCG (Fig. 7). The processing was inhibited by the Zn^{2+} chelator 1,10 *O*-phenanthroline (1 mM) and by the inhibitor of the metalloproteinases TIMP2 (100 ng/ml), but not by the inhibitor of the serine proteases phenylmethylsulfonylfluoride (1 mM). Treatment of the cells with HbA decreased the level of the MMP-2 and its activation in CD-treated monolayers. In the data presented here, no gelatino-lytic activity was detected in HbA treated cells in RCG, but a similar analysis performed using concentrated conditioned medium demonstrated a nine-fold reduction of MMP-2 activation (not illustrated). Blocking PKC with BIM had no effect.

3. DISCUSSION

Control of the phenotype of connective tissue cells by mechanical forces is a meaningful regulatory mechanism involved in the adaptation of the extracellular matrix (ECM) to its supporting function. Our data demonstrate that a panel of matrix metalloproteinases (MMPs) participating in the degradation of the various components of the ECM are up-regulated or activated by stress-relaxation obtained either by disruption with cytochalasin D of the active cellular mechanism generating mechanical tension, the ac-tomyosin fibers, or by dissipation of the tension forces upon retraction of a free floating collagen gel (RCG). The present data complement previous studies specifically investigating MMP-1 up-regulation by stress relaxation (Lambert et al., 1992, 1998). The present study was performed by a RT-PCR procedure made quantitative by adding, in each sample, a synthetic RNA as internal standard, co-transcribed and co-amplified with the cellular RNA, using the same primers under non-competitive conditions (Freeman et al., 1999). This procedure provided comparative values allowing to define, for each MMP, a pattern of reactivity to stress relaxation and to investigate some signaling pathways involved in their regulated expression.

While a large increase of MMP-1, -3, -9, -13 and -14 expression was observed in both conditions of stress relaxation (CD and RCG) (see also Unemori and Werb, 1986; Mauch et al., 1988,1989; Lambert et al., 1992, 1998; Ravanti et al., 1999), the expression of MMP-2 was less modulated. The up-regulation induced by CD was always larger than that observed in RCG. This might be related to the extensive disruption of the cytoskeleton and focal adhesions by CD (Miyamoto et al., 1995) while the integrin-mediated retraction of the collagen gel results in reduced alterations of the cytoskeleton (Chicurel et al., 1998).

As previously observed for MMP-1 in human (Lambert et al., 1998) and rabbit fibroblasts (West-Mays et al., 1995), the up-regulation induced by relaxation with CD is suppressed by CHX for all the MMPs investigated here. The RCG-induced up-regulation of MMP-2 and MMP-13 is also suppressed by CHX. These observations strongly suggest that these up-reg-ulations depend, at least in part, on de novo protein synthesis. CD has been shown to induce MMP-1 expression in rabbit corneal fibroblasts through the activation of an autocrine loop of IL-1α (West-Mays et al., 1997). This signaling pathway is, however, not functional in human fibroblasts for MMP-1 and can not be considered for MMP-13 since IL-1 inhibits its expression (Ravanti et al., 1999). Although not reaching statistical difference, due to the low level of expression and the variability of the results, we have observed an increase of MMP-9 mRNA level by blocking protein synthesis in RCG while CHX largely decreased the up-regulation induced by CD. This suggests that stress relaxation by CD leads to the synthesis of factors acting positively on MMP-9 mRNA production or stability, while in RCG the level of this mRNA is under the control of negatively intervening factors.

The induction of MMP-3, -9, -13 and -14 upon stress relaxation by CD treatment, and that of MMP-3 and -13 in RCG, is suppressed or reduced by the tyrosine kinase inhibitor HbA, suggesting a role for one or several members of the src family in these regulations, although the specificity of HbA toward the src-kinases is not absolute (Uehara and Fukazawa, 1991). In this context, it is worth noting that the transient expression of v-src induces MMP-1 expression in rabbit synovial fibroblasts (Vincenti et al., 1996). Furthermore, the genetic suppression of src in mice decreases bone degradation resulting in an osteopetrotic phenotype (Soriano et al., 1991). More recently, it was shown that cells harboring null mutations of Src and the related kinases Fyn and Yes were deficient in integrin-mediated signaling (Klinghoffer et al., 1999).

As previously described for MMP-1, the inhibition of PKC by BIM reduced the CD-induced expression of MMP-2, -3 and -9, but not the RCG-induced up-regulation, suggesting that CD and RCG use different signaling pathways. The MMP-13 expression, which is reduced by BIM in CD-treated monolayer, is largely up-regulated by the same inhibitor in RCG. At the concentration used here, BIM inhibits the activity of the TPA-dependent isoforms of PKC, showing that the signaling pathways induced by CD, but not in RCG, involves such PKC. However, it cannot be excluded that TPA-independent PKC isoforms, not inhibited by BIM at this concentration, are involved in the up-regulation of the MMPs in RCG. Xu and Clark (1997) have indeed shown that PKC-ζ is activated in RCG and participates in the up-regulation of the MMP-1. Other authors (Sudbeck et al., 1994; Vincenti et al., 1996) and ourselves (Lambert et al., 1998) have also demonstrated that TPA-induced expression of MMP-1 is inhibited by tyrosine kinase inhibitors, suggesting that PKC may act upstream of Src in the up-regulation of the MMPs by phorbol esters and CD, and possibly also in RCG.

The ERK1,2, JNK and p38 MAP kinases pathways in fibroblasts are rapidly activated in RCG (Ravanti et al., 1999). According to Ravanti et al. (1999), the p38 MAP kinase is essential for the induction of MMP-13 in RCG, while ERK1,2 mediate its repression. A similar conclusion was made for the CD-treated monolayer. By contrast p42/44 and p38 MAP kinases participate in the induction of MMP-3 in CD-treated monolayers. p38 MAP kinase also participates in the MMP-9 induction by CD while it triggers its repression in RCG. These data point to the divergent regulation of various MMPs by the ERK1,2 and the p38 MAP kinase pathways.

The promoters of MMP-1, -3, -9 and -13 have in common: a TATA-box; a proximal AP-1 binding site; and additional AP-1 and Ets-family proteins binding sites more upstream (Benbow and Brinckerhoff, 1997). The proximal AP-1 site plays a major role in the basal transcription of the genes and their transactivation by TPA and cytokines. Mutational analysis has, however, demonstrated the participation of distal *cis*-acting elements in this regulation. MMP-9 also contains distal SP-1 and NF-κB sites, both of which are important for promoter activity (Huhtala et al., 1991; Sato and Seiki, 1993; Bond et al., 1998). By contrast, MMP-2 and -14 promoters are TATA-less (Huhtala et al., 1990; Lohi et al., 2000), contain proximal SPl-se-quence(s) and are devoid of AP-1, Ets or NF-κB sites. The moderate modulation of MMP-2 gene expression by CD and RCG and the lack of effect of BIM on MMP-14 up-regulation may reflect the absence of AP-1 sites in the promoter of these two MMPs. The participation of AP-1 site(s) in the over-expression of MMP-1, -3, -9 and -13, induced by CD and in RCG, needs further investigation. Its (their) activity is, however, suggested by the induction of c-Fos and c-Jun by the abrupt release of stress in retracting collagen gels (Rosenfeldt et al., 1998) and the induction of c-Fos by CD in

HeLa and WI-38 cells (Zambetti et al., 1991).

The control of MMP-2 activity largely depends on the processing of the latent proenzyme rather than modulation of its expression. This activation is performed by various members of the membrane-associated (MTl-MT5)-MMPs. Besides a moderate increase in expression, MMP-2 is almost completely processed into an active form by CD treatment and culture in RCG, which is in agreement with previously published data (Seltzer et al., 1994; Tomasek et al., 1997). This effect is suppressed by TIMP2 and the Zn^{2+}-chelator 1,10 *O*-phenanthroline, but not by PMSF, a serine-protease inhibitor, suggesting that activation is performed by members of the MMP family. MT-2 and MT-3 MMPs (MMP15 and 16) are not expressed in the fibroblasts used here (personal observation). We suggest that MMP-14 (MT1-MMP), which is largely up-regulated by CD and in RCG, participates in the maturation of the MMP-2, as it was demonstrated by using MMP-14 blocking antibodies for melanoma cell lines grown in RCG (Kurschat et al., 1999). This hypothesis is consistent with the finding in our experimental models that MMP-2 activation can be correlated with MMP-14 over-expression. However, we cannot exclude a participation of MT-4 and MT-5 MMP. Surprisingly, HbA decreased the amount of MMP-2 secreted by CD-treated cells and in RCG without affecting its mRNA level, suggesting a translational or post-translational regulation.

Contrasting to MMP-2, MMP-9 was not detected in any sample by ELISA or by zymography even after a 10-fold concentration of the conditioned mediums. This observation correlates with the fact that MMP-2 mRNA is detected after 25 cycles of amplification while MMP-9 mRNA requires 38 cycles. Although MMP-9 expression is probably too low to be biologically significant, its regulation was studied here for comparative purposes.

The up-regulation of the panel of MMPs involved in the degradation of the ECM components, by integrin-mediated stress relaxation of the resident fibroblasts, participates in an adaptative process of remodeling that may be significant in developmental, physiological and pathological processes. The specificity of the signaling pathways regulating their expression supports the distinct role played by these proteases.

4. EXPERIMENTAL PROCEDURES

4.1. Culture, chemicals and experimental procedure

Human skin fibroblasts, isolated and cultured as previously described (Delvoye et al., 1991), were plated on polymeric bovine type I collagen-coated dishes, or included in freely floating retracting collagen gels (RCG), as described earlier (Lambert et al., 1992). Treatments with the various inhibitors were performed as detailed in Section 2. Tetradecanoyl phor-bol acetate (TPA); herbimycin A (HbA); cytochalasin D (CD); 1,10 *O*-phenanthroline (Sigma, St. Louis, MO); PD98059; SB 203580 (Alexis Biochemicals, Lausanne, Suisse); and bisindoyl maleimide (BIM) (Roche Molecular Biochemicals, Mannheim, Germany) were prepared as stock solutions in dimethyl-sulfoxide. Y-27632 (a generous gift from Dr Yoshimura, Welfide Co., Osaka, Japan) was solubilized in water. Human recombinant tissue inhibitor of metalloproteinase TIMP2 was a generous gift from N. Sounni (Laboratory of Tumor and Development Biology, Liège, Belgium).

4.2. Tissues and RNA preparation

Mouse neutrophils were enriched from blood sample by centrifugation through Lymphoprep (Nycomed Pharma, Oslo, Norway). Human neutrophils were received from Dr J. Pincemail. Human and mouse placentas were obtained at, respectively, the third trimester and the third week of gestation. Total RNA from RCG and placenta was purified by ultracentrifugation on a cesium chloride cushion (Chirgwin et al., 1979) and that from neutrophils and cells in monolayer culture by the same technique, or by using the High Pure RNA Isolation kit (Roche Molecular Biochemicals). RNA concentrations were determined by measuring OD at 260 nm.

4.3. RT-PCR

The RT-PCR reactions were performed in an automated thermal cycler (GeneAmp PCR System 2400 or 9600, Perkin-Elmer, Norwalk, CT) using: the GeneAmp Thermostable rTth Reverse Transcriptase RNA PCR kit (Perkin-Elmer); specific pairs of primers (5 pmoles each, Eurogentec, Liège, Belgium); 10 ng of total RNA; and a known copy number of an original synthetic internal standard RNA constructed as described in Section 2, in 25-µl reaction mixture. The RT step (70°C for 15 min) was followed, after a 2-min incubation at 95°C for denaturation of RNA/DNA heteroduplexes, by PCR amplification for the adequate number of cycles and terminated by a final elongation step of 2 min at 72°C. The PCR conditions for amplification of MMP-2, -8, -9 and -14 and the 28S rRNA were: 94°C for 15 s; 66°C for 20 s; and 72°C for 10 s. For MMP-3, -11 and -13 the

conditions were: 94°C for 15 s; 63°C for 30 s; and 72°C for 30 s. The RT-PCR products were resolved in 10% polyacrylamide gel and analyzed using a Fluor-S-MultiImager (BioRad, Hercules, Ca) after staining with GelStar (FMC BioProducts, Rockland, ME) dye.

4.4. Zymography and ELISA

Monolayers were rinsed three times and the collagen gels washed three times for 15 min in Dulbecco's and incubated for 24 h in serum-free culture medium. Aliquots of the conditioned culture mediums, corresponding to a defined number of cells as determined by DNA measurements, were used for analyzing the secreted MMP-2 by zymography as previously described (Sakalihasan et al., 1996). In some experiments, the conditioned mediums were concentrated by lyophilisation after dialysis against water. MMP-9 was assayed by ELISA (R and D Systems, Abingdon, UK) as described by the manufacturer and by zymography.

4.5. Statistical analysis

The results are expressed as the mean values ± standard deviation. The statistical analysis was performed using Student's f-test. The number of experiments is indicated in the figure legends.

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