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Convenient fluorometric assay for matrix metalloproteinase activity and its application in biological media

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Abstract Matrix metalloproteinases (MMPs) are involved in physiological tissue remodeling and pathological conditions like tumour metastasis and joint destruction. Until now, no convenient and sensitive MMP-activity assay in crude media like synovial fluid has been available. Therefore, the highly soluble fluorogenic substrate TNO211 (Dabcyl-Gaba-Pro-Gln-Gly-Leu-Glu(EDANS)-Ala-Lys-NH_2), containing the MMP cleavable Gly-Leu bond and EDANS/Dabcyl as fluorophore/quencer combination, was synthesized and characterized as an MMP specific substrate. We show that the fluorogenic assay using TNO211 is sensitive and can detect MMP activity in culture medium from endothelial cells and untreated synovial fluid (SF) from RA and OA patients, and control subjects. MMP activity in SF significantly increased in the order C < OA < RA, thus the frequent use of OA samples as control in studies on RA is debatable.

Key words: Matrix metalloproteinase; Synovial fluid; Fluorescence quenching

1. Introduction

Matrix metalloproteinases (MMPs; e.g. collagenases, stromelysins and gelatinases) are involved in the degradation of the extracellular matrix in physiological and pathophysiological turnover of tissues. In particular, elevated levels of MMPs have been found in pathological conditions such as rheumatoid arthritis, osteoarthritis [1], and tumour metastasis [2,3]. After excretion from the cell as inactive pro-enzyme (proMMPs), MMPs undergo extracellular activation into the active enzyme, which can be rapidly neutralized by reversible, stoichiometric binding with tissue inhibitors of metalloproteinases (TIMPs). Therefore, to assess the potential of matrix degradation by MMPs (levels of active MMP are usually less than 5% of total MMP), net enzyme activity is more appropriate than total amounts of (pro)MMPs plus MMP-TIMP complexes as frequently determined by immunoassay [4–7]. Activity assays from MMPs comprise methods based on physiological substrates (e.g. radiolabeled collagen, casein or proteoglycans) or synthetic substrates. The former methods are mostly too insensitive for detection of MMP activity in unactivated biological media. Recently, the use of fluorogenic peptides for measuring activity of purified MMPs was described [8–11]. These substrates consist of a fluorophore and a light-absorbing group (quencher) attached to an amino acid sequence that is recognized by MMPs. After enzymatic cleavage the quenching is eliminated and fluorescence is observed. So far, the use of this kind of substrates in synthetic MMP-inhibitor studies or biological media is hampered by optical disturbances from the medium and their low solubility, due to the (lipophilic) fluorophores and quenchers used.

The present study was designed to develop a water soluble fluorogenic MMP substrate containing the charged EDANS/Dabcyl group [12] and apply it to relatively complex samples such as cell culture medium and synovial fluid.

2. Materials and methods

2.1. Synthesis of fluorogenic substrate TNO211

The fluorogenic substrate Dabcyl-Gaba-Pro-Gln-Gly-Leu-Glu (EDANS)-Ala-Lys-NH_2 was synthesized according to the method described by Drijfhout et al. [13].

2.2. Proteolytic enzymes

The gelatinase zymogens pro-MMP-2 [14] (EC 3.4.24.24) and pro-MMP-9 [15] (EC 3.4.24.35) were kindly provided by Dr. H. Nagase (University of Kansas, USA) and Dr. V. Knäuper (Strangeways Laboratories, Cambridge, UK), respectively. Recombinant human stromelysin [11] (active form, MMP-3; EC 3.4.24.17) and the 19 kDa catalytic domain of collagenase [16,17] (19 kDa MMP-1; EC 3.4.24.7) were a gift from Dr. J. McGeehan (Glaxo Inc., Research Triangle Park, NC, USA). The 19 kDa MMP-1 has the same specific activity for small peptide substrate as full length MMP-1 [17,18] and was therefore considered suitable for the present study. Pro-MMP-2 was activated at 37°C for 2 h and pro-MMP-9 at 4°C for 18 h in buffer A (50 mM Tris (pH 7.6), 150 mM NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.01% Brij 35) plus 1 mM APMA, resulting in >90% activation into the active enzymes (validated with gelatin zymography [19]). Plasmin (EC 3.4.21.7) was obtained from Kordia (Leiden, the Netherlands), human recombinant urokinase-type plasminogen activator (u-PA, EC 3.4.21.73), from Serono (Aubonne, Switzerland) and human neutrophil elastase (EC 3.4.21.37) from the Bloodbank (Leiden, the Netherlands); all these enzymes were supplied in the active form.

2.3. Incubation conditions

All incubations were performed at 37°C in buffer A, unless stated otherwise. Enzyme and substrate were preincubated separately for

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Abbreviations: APMA, 4-aminophenylmercuric acetate; Cha, cyclohexylalanine; Cys(Me), *S*-methylcysteine; Dabcyl, 4-(4-methylaminophenylazo)benzoyl; Dnp, 2,4-dinitrophenyl; Dpa, *N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; EDANS, 5-((2-aminoethyl) amino)naphthalene-1-sulphonic acid; KIU, kallikrein inhibitor units; Mca, (7-methoxycoumarin-4-yl)acetyl; MMP, matrix metalloproteinase; Nma, *N*-methylanthranilic acid; RP-HPLC, reversed-phase high-performance liquid chromatography; RS-47,112, [4-(*N*-hydroxyamino)-2*R*-isobutyl-3*S*-(thiopen-2-ylthiomethyl)succinyl]-L-phenylalanine-*N*-methylamide; TFA, trifluoroacetic acid; TOF-MALDI, time-of-flight matrix-assisted laser desorption-ionisation; u-PA, urokinase-type plasminogen activator.

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5 min at 37°C. Inhibitors Na_2EDTA and aprotinin (Kordia, Leiden, The Netherlands) were dissolved in buffer A. A stock solution (10 mM) of the MMP inhibitor RS-47,112 (gift of Dr. R. Martin, Roche Bioscience, Palo Alto, CA, USA; also known as BB-94 [20]) was made in DMSO. Conversion of TNO211 by plasmin, u-PA, or elastase was investigated by incubation of 5 μM substrate with high concentrations of these enzymes (100 nM) in buffer A by monitoring fluorescence (λ_{ex} = 340 nm, λ_{em} = 485 nm; Kontron SFM-25 fluorimeter).

2.4. Solubility of fluorogenic substrates

Saturated solutions of 50 nmol peptide in 100 μl incubation buffer A were made without the addition of organic solvents. After 1 h at 37°C and 5 min of vortexing, the samples were centrifuged (5 min at 10 000 × *g*) to remove insoluble material, and were subjected to RP-HPLC to determine the solubility of the substrate. Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 was from Bachem (Bubendorf, Switzerland) and DNP-Pro-Cha-Gly-Cys(Me)-His-Ala-Lys(Nma)-NH_2 was a gift from Dr. J. McGeehan [11].

2.5. Reversed phase-high performance liquid chromatography (RP-HPLC)

Purification of synthesized peptide and quantitation of cleavage products was determined with a Perkin-Elmer C_{18} Pecosphere 3×3C column eluted with a linear acetonitrile gradient in 0.1% TFA; 1.0 ml/min delivered by a Gynkotek Model 480 pump. Samples were injected with a WISP model 710B automated injector (Waters); detection comprised absorbance at 214 or 520 nm (Applied Biosystems Model 759A) and a Jasco Model 821-FP fluorescence detector (λ_{ex} = 340 nm, λ_{em} = 485 nm). Data analysis was performed with Peakmaster v3.2 (Harley Systems, Bucks, UK). Identity of the substrate and cleavage products was established by TOF-MALDI mass spectrometry [21] (Lasermat, Finnigan MAT) and amino acid analysis of collected fractions [22].

2.6. Enzyme kinetics

Proteolytic enzymes (19 kDa MMP-1, 25nM; MMP-2, 1.5 nM; MMP-3, 7.2 nM; MMP-9, 1.5 nM) were incubated with 1.8 μM TNO211 in buffer A. At 0, 20, 40, 60 and 90 min, 50 μl aliquots were added to an equal volume of ice-cold 40 mM EDTA in 0.1 % TFA. Substrate concentrations at time point zero, $[S]_0$, and other time points, $[S]_t$, were quantified by RP-HPLC. Catalytic efficiencies (k_{cat}/K_m values) were calculated from the equation $[S]_t/[S]_0 = e^{-kt}$, with $k = [E]_{tot} \cdot (k_{cat}/K_m)$ [11,23].

2.7. MMP activity in synovial fluid and culture medium

The feasibility of applying EDANS/Dabcyl peptides to determine MMP activity was investigated with synovial fluid and tissue culture samples. Synovial fluids were obtained from patients with rheumatoid arthritis (RA-SF; n = 8, mean age 48 years, range 32–73 years), patients with osteoarthritis (OA-SF; n = 5, mean 66 years, range 52–80 years), and post mortem from individuals without any sign of arthritis, which were regarded as controls (C-SF; n = 9, mean 45 years, 23–76 years). After centrifugation (4°C, 10 000 × *g*, 15 min) and 5-fold dilution with incubation buffer A, MMP activity of 75 μl diluted SF (without activation of proMMPs) was measured at 37°C with substrate TNO211 (5 μM) in a total volume of 150 μl incubation buffer. Increase in fluorescence was followed continuously (λ_{ex} = 340 nm, λ_{em} = 485 nm; Kontron SFM-25 fluorometer). Incubation time was extended up to 6 h for samples containing low activity to acquire reliable ΔRFU/time data. Conditioned media (75 μl) from untreated human umbilical vein endothelial cells cultured under serum-free conditions for 24 h [19] were incubated with 5 μM TNO211 in a total volume of 150 μl. Increase in fluorescence was measured as mentioned above. To some incubations of biological media enzyme inhibitors were added (see figure legends for details).

3. Results

3.1. Substrate cleavage site

Substrate TNO211 (Dabcyl-Gaba-Pro-Gln-Gly-Leu-Glu (EDANS)-Ala-Lys-NH_2) was incubated with all MMPs available and hydrolysis was followed by RP-HPLC to confirm that cleavage occurred exclusively at the Gly-Leu bond: incubation of substrate TNO211 with MMP-9 results in two

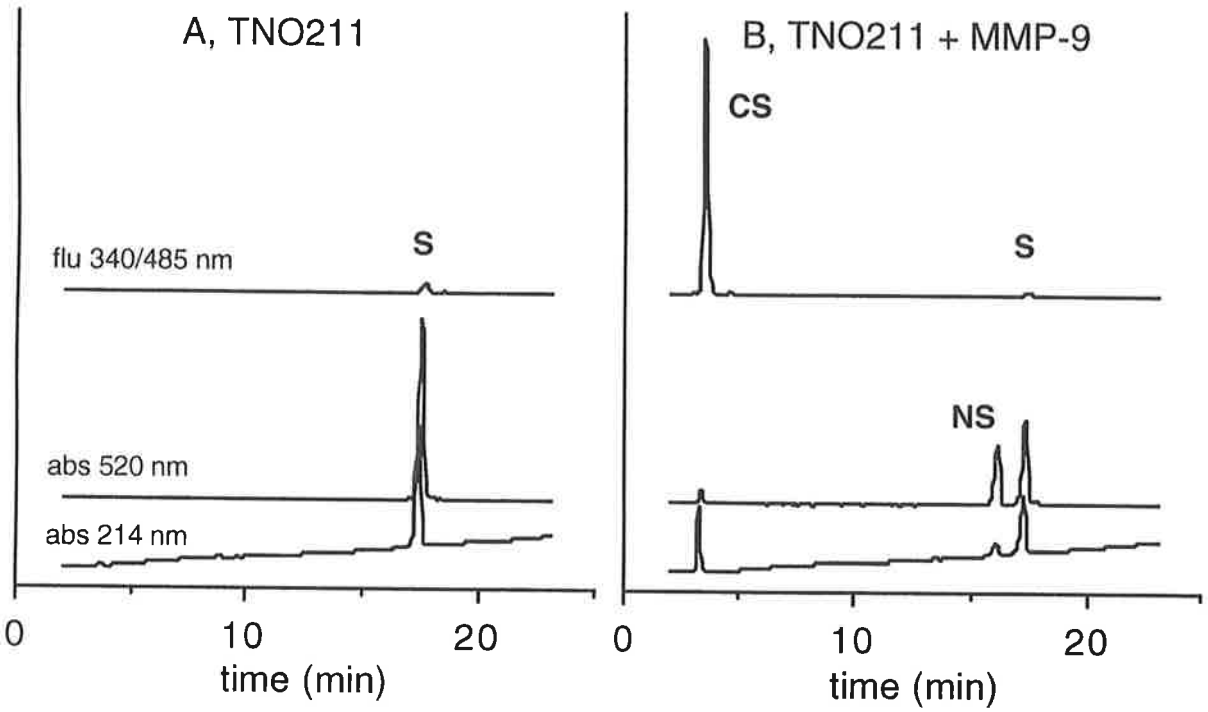

Fig. 1. Cleavage of TNO211 by MMP-9. HPLC analysis of incubation mixtures of TNO211 in the absence or presence of MMP-9: S, the parent peptide Dabcyl-Gaba-Pro-Gln-Gly-Leu-Glu(EDANS)-Ala-Lys-NH_2 (MH^+_{meas} = 1327.6, MH^+_{calc} = 1326.5); NS, the N-terminal fragment (Dabcyl-Gaba-Pro-Gln-Gly; MH^+_{meas} = 638.3, MH^+_{calc} = 637.3; CS, the C-terminal cleavage product (Leu-Glu(EDANS)-Ala-Lys-NH_2). Note the quenched fluorescence of the parent peptide. Small differences in retention times correspond to void volumes between detectors.

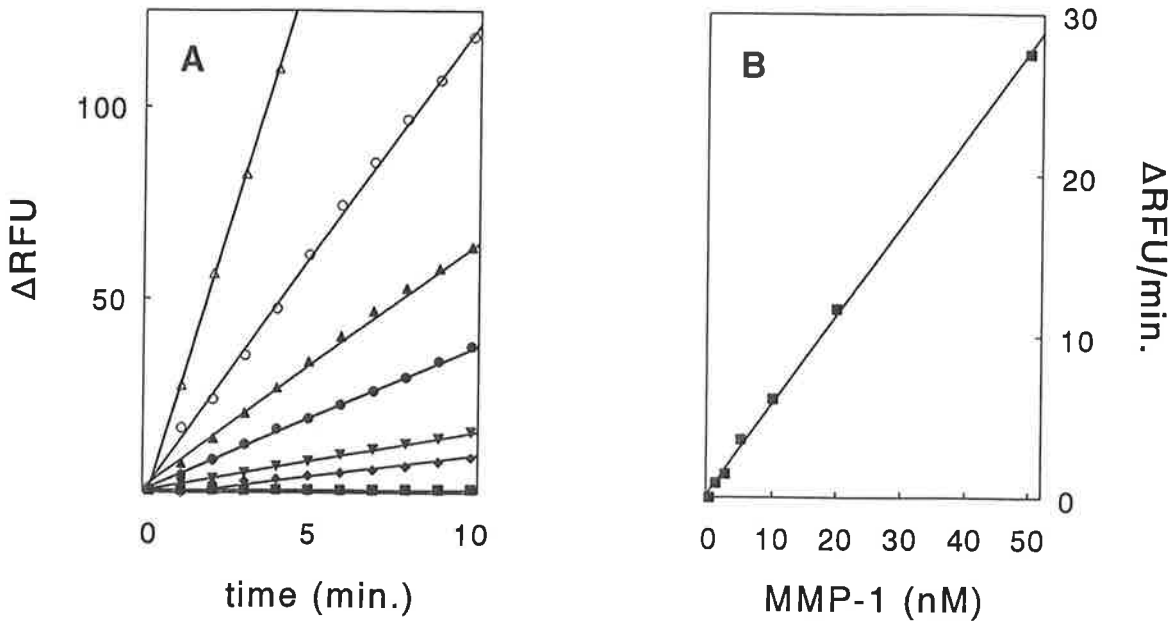

Fig. 2. Time and enzyme dependence of the turnover of TNO211. Change in fluorescence (ΔRFU) increased linearly with time upon incubation of TNO211 (5 μM) with MMP-1 (△, 50 nM; ○, 20 nM; ▲, 10 nM; ●, 5 nM; ▼, 2.5 nM; ◆, 1 nM). No change in fluorescence was observed with buffer only (■), or a high concentration of MMP-1 (50 nM) in the presence of 10 mM EDTA (◇; coincides with the data of the substrate control). The rate of change in fluorescence (ΔRFU/time) derived from the lines in (A) was proportional to the concentration of MMP-1 (B).

new peaks (at 214 nm) in the chromatogram, with a concomitant decrease in peak height of the substrate peak (Fig. 1). The first peak also exhibited fluorescence. Using amino acid analysis , this peak was identified as the C-terminal hydrolysis product Leu-Glu(EDANS)-Ala-Lys-NH_2. Detection at 520 nm (Dabcyl group) showed two major peaks in the chromatogram: the parent compound (S) and the N-terminal cleavage product Dabcyl-Gaba-Pro-Gln-Gly (NS). The identity of the latter was confirmed by amino acid analysis and mass spectrometry ($MH^+_{meas} = 638.3$). This exclusive cleavage of the Gly-Leu bond was found for all the MMPs tested.

3.2. *Fluorescence response of EDANS/Dabcyl peptides*

To establish whether measurement of fluorescence is a reflection of turnover of the synthetic substrates, the fluorescence was related to the amount of cleavage product formed (measured by HPLC). Incubation of TNO211 with MMP-9 showed that fluorescence values are proportional to the amount of N-terminal cleavage product, Dabcyl-Gaba-Pro-Gln-Gly ($r > 0.999$) and EDANS fluorescence being quenched 98.4% by Dabcyl. Furthermore, the rate of increase in fluorescence was linearly related to the amount of enzyme ($r > 0.99$; 5 μM TNO211, Fig. 2). TNO211 allows sensitive measurement of MMP activity: for instance, 1 nM MMP-1 can routinely be measured in a 10 min incubation with purified enzyme (Fig. 2). Substantially lower detection limits (down to 10 pM) can simply be achieved by increasing the incubation time.

3.3. *Enzyme kinetic properties and solubility of EDANS/Dabcyl peptides*

For all TNO211-MMP combinations tested, first order enzyme kinetics were observed: incubations showed a linear decrease of $\ln([S]_t/[S]_0)$ as a function of time ($r > 0.99$). Catalytic efficiencies k_{cat}/K_m, ranged from 21 000 (MMP-1) to 619 000 M^{-1} s^{-1} (MMP-2, see Table 1). Fluorogenic substrates studied by others show large differences in k_{cat}/K_m values between the different MMPs [8,9]. For instance, two commercially available fluorogenic substrates, Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 [9] and DNP-Pro-Leu-Gly-Leu-Trp-Ala-*D*-Arg [8] show a 40–70-fold higher k_{cat}/K_m for MMP-2 (even at 25°C) than for MMP-1 (at 37°C; Table 1). For substrate TNO211, this ratio between MMP-2 and

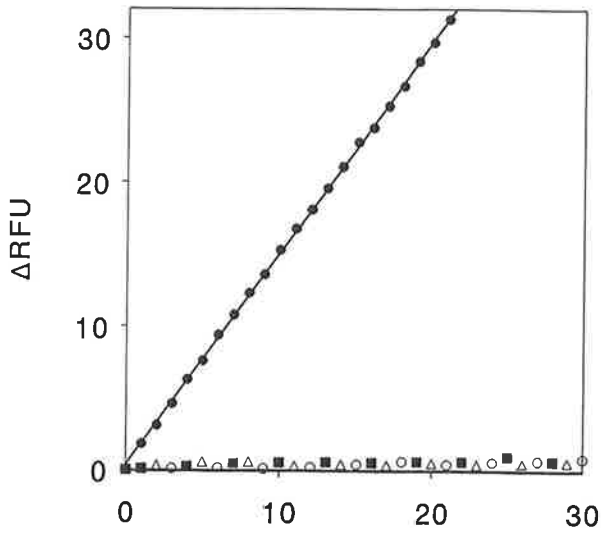

Fig. 3. Hydrolysis of EDANS/Dabcyl substrates by MMP-1, plasmin, u-PA, or elastase. TNO211 (5 μM) was incubated at 37°C with 100 nM plasmin (■), u-PA (△), or elastase (○). No change in fluorescence was observed. In contrast, a substantially lower concentration of MMP-1 (●, 1 nM) resulted in a rapid increase in fluorescence.

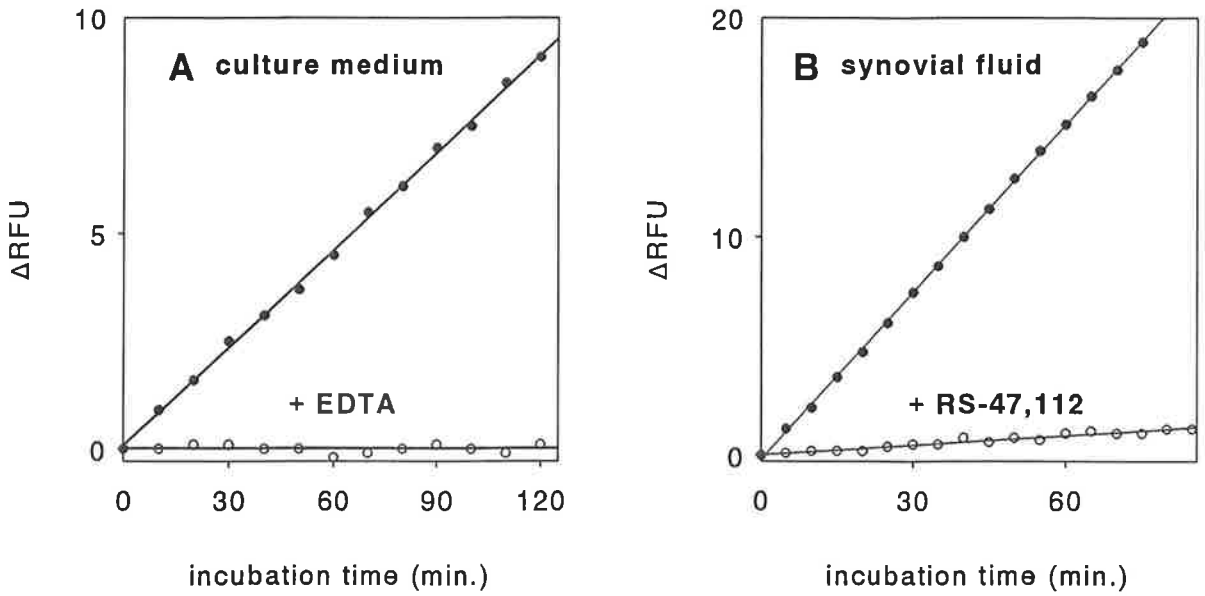

Fig. 4. MMP enzyme activity in unactivated culture medium or synovial fluid. Unactivated culture medium (A) or synovial fluid (B) was incubated with substrate TNO211 (5 μM; 37°C). Conditioned medium from human umbilical vein endothelial cells (serum-free; 2-fold diluted, ●) resulted in increasing fluorescence (ΔRFU), linearly with time. This increase in fluorescence was totally abolished by 10 mM EDTA (○, A). Fluorescence resulting from the cleaved fluorogenic substrate increased linearly with time in the presence of synovial fluid from a rheumatoid arthritis patient (10-fold diluted, ●). Addition of the synthetic MMP-inhibitor RS-47,112 (also known as BB-94; 10 μM) suppressed the change in fluorescence effectively (○, B).

MMP-1 (both determined at 37°C) is only 29. The catalytic efficiency of TNO211 for MMP-3 is 2-fold greater than that for MMP-1, similar to Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (1.6-fold). Altogether, TNO211 represents a general MMP substrate. Furthermore, TNO211 was highly soluble (80 μM) in buffer without the addition of organic solvents, in contrast to MMP substrates containing Mca/Dnp or Nma/Dnp as fluorophore/quencher couple (less than 5 μM, Table 1). In synthetic MMP-inhibitor studies where substrate concentrations up to 3 times K_m are preferable (estimated for TNO211, $K_m = 25$ μM, and for Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2, $K_m = 70$ μM, both for MMP-2 [9]), substrate TNO211 is favoured over the lipophilic substrates containing Mca/Dnp or Nma/Dnp.

3.4. Specificity of EDANS/Dabcyl peptides for MMPs

In biological fluids and culture media proteinases other than MMPs can be abundant like the serine proteinases plasmin and u-PA, and the metalloproteinase elastase [24,25]. Cleavage of the EDANS/Dabcyl substrates by these enzymes will interfere in an MMP-activity assay. Therefore, incubations were performed with fluorogenic substrate TNO211 and 100 nM plasmin, u-PA or elastase. The increase in fluorescence was followed in time and compared to the hydrolysis by 100-fold lower concentrations of MMP-1 (1 nM). No conversion of the substrate by plasmin, u-PA or elastase was detected after 30 min, whereas increase in fluorescence with as low as 1 nM MMP-1 could easily be monitored (Fig. 3). Furthermore, addition of 10 mM EDTA (Fig. 4) or 1,10-phenanthroline totally abolished the conversion of substrates. In conclusion, interference by elastase or serine proteinases in MMP-activity assays in crude media with TNO211 is unlikely.

3.5. MMP activity in culture medium and synovial fluid

Although several fluorogenic substrates for MMPs have been described, their application to measure MMP activity in biological media has not yet been reported or failed [8] due to optical interactions of the fluorophore with the medium. Since the emission wavelength of EDANS is relatively high (485 nm) in comparison to other fluorophores used in fluorogenic MMP substrates, disturbances from the medium will be less severe.

Serum-free medium from non-activated human endothelial cells have been shown to contain MMPs (predominantly gelatinases [19]). When incubated with TNO211, a linear increase in fluorescence was observed within minutes, indicating

Table 1
MMP substrate: amino acid sequence, solubility and catalytic efficiency (k_{cat}/K_m) at 37°C

	Substrate	Solubility[a] (μM)	k_{cat}/K_m ($\times 10^3$) (M^{-1} s^{-1})				Ref.
			MMP-1	MMP-2	MMP-3	MMP-9	
TNO211	Dabcyl-Gaba-Pro-Gln-Gly▼Leu-Glu(EDANS)-Ala-Lys-NH_2	80	21	619	40	206	
	Dnp-Pro-Leu-Gly▼Leu-Trp-Ala-D-Arg-NH_2	N.D.	0.83	58[b]	2.2		[8]
	Mca-Pro-Leu-Gly▼Leu-Dpa-Ala-Arg-NH_2	4.3	14.8	629[b]	23		[8]
	Dnp-Pro-Cha-Gly▼Cys(Me)-His-Ala-Lys(Nma)-NH_2	4.6	13			86.6	[11]

▼, MMP cleavage site; N.D. not determined.
[a]Solubility in buffer A without addition of organic solvents;
[b]Determined at 25°C.

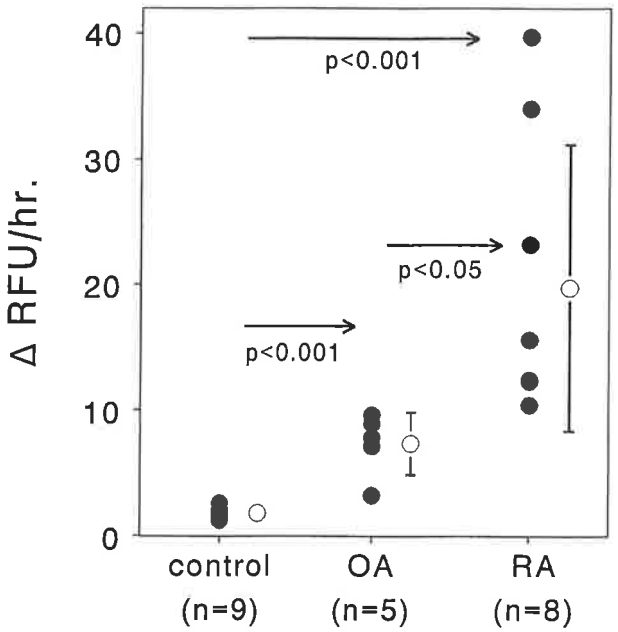

Fig. 5. MMP enzyme activity in synovial fluid from controls, osteoarthritis and rheumatoid arthritis patients. MMP activity in synovial fluids measured with TNO211. Closed symbols are individual data, open symbols represent the group mean ± S.D. See Section 2 for incubation details. Student's *t*-test was used for statistical analysis.

the presence of active MMPs in the conditioned medium. The addition of EDTA totally suppressed the increase of fluorescence (> 99%; Fig. 4A). Also, unactivated synovial fluid from rheumatoid arthritis patients caused a linear increase in fluorescence with time (Fig. 4B). This increase in fluorescence could be inhibited 94% by 10 μM RS-47,112 (a synthetic MMP inhibitor [20]) or 10 mM EDTA (95% inhibition). As expected, no inhibition was found when 10 μM (400 KIU/ml) aprotinin, a serine proteinase inhibitor, was added to the incubation. These experiments indicate that only MMPs in SF are responsible for the turnover of the fluorogenic substrate TNO211. Synovial fluids from patients with osteoarthritis and from patients with rheumatoid arthritis showed significantly higher MMP activities with TNO211 than control SF, indicating increased levels of active MMPs in pathological situations (Fig. 5). Although activities in control subjects were low, enzymatic activity could be reliably quantified by extending incubation time up to 6 h. Since MMP activity was significantly increased in OA vs. control SF, the frequent use of osteoarthritis patients as controls in studies with respect to MMPs on rheumatoid arthritis can be debated and merits further investigation.

4. Discussion

The present study shows that synthetic peptides with EDANS and Dabcyl groups are efficiently hydrolyzed by MMPs. TNO211 seems a good substrate in enzyme kinetic studies or inhibitor studies because of its high solubility. Furthermore, the fluorescence properties of the substrate allow convenient measurement of MMP activity in complex biological media like synovial fluid and culture medium. Since active MMP is detected only, this type of assay will be more useful than immunological methods where antibodies also recognize inhibited forms of MMP [4–7]. MMP activity in SF could be indicative for joint destruction and therefore used as a marker in prognostic, therapeutic or diagnostic studies. However, we realize that the fairly general substrate TNO211 is not ideal to gain insight in the role of specific MMPs in physiological and pathological types of tissue remodeling. Therefore, new EDANS/Dabcyl substrates selective for classes of MMPs are presently under investigation.

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