Effect of PEP1261 on expression of MMP-2 in presence of nitric oxide in synovial fibroblasts from rats with CIA
INTRODUCTION

Matrix metalloproteinases (MMPs) constitute a family of zinc-dependent proteolytic enzymes with so far 28 identified members (Visse and Nagase, 2003). MMPs, capable of degrading several components of extracellular matrix (ECM), are thought to be implicated in pathological processes of inflammatory joint diseases (Unemori et al., 1991). Overexpression of MMPs in synovia and elevated serum levels of MMPs have been demonstrated in rheumatoid arthritis (RA) patients (Walakovits et al., 1992; Wolfe et al., 1993). MMP regulation occurs at the level of gene transcription and activation of proenzymes. Several biological mediators have been noted to induce the expression of MMPs (Matrisian, 1992).

Nitric oxide (NO) is also related to the pathophysiology of RA and it plays an important role in cartilage catabolism (Farrell et al., 1992). NO is synthesized via L-arginine oxidation by nitric oxide synthases (NOS) (Moncada and Higgs, 1993). NOS isoforms are either calcium-dependent that are constitutively expressed (cNOS and eNOS) or calcium-independent inducible enzymes (iNOS). cNOS generates physiological amounts of NO and it plays a role in physiological function, such as vascular reactivity (Nathan and Xie, 1994). In contrast, iNOS generates large amounts of NO and it contributes to the pathogenesis of inflammatory diseases (Moncada et al., 1991). The appearance of iNOS is usually associated with the generation of high levels of nitric oxide, which in turn form ROS (Porasuphatana et al., 2003). In inflammation, ROS play a special role as signalling
molecules which contribute to cell injury and degenerative processes such as cartilage degradation in rheumatic diseases (Schiller et al., 2003).

Interestingly, MMPs can be activated by ROS (Belkhiri et al., 1997) so that both seem to contribute vitally to the inflammatory network. For the activation of MMPs by ROS, various intracellular signalling pathways have been identified (Yoon et al., 2002). It has already been shown that PEP1261 has excellent radical scavenging properties (Meera et al., 1999) and it enhances the production of antioxidative enzymes in adjuvant-induced arthritis (Ashok Kumar et al., 2002), which contribute to the anti-inflammatory effect of the peptide.

In this study, the role of the tetrapeptide derivative PEP1261 \{Boc-Lys(BOC)-Arg-Asp-Ser(tBu)-OtBu\}, a peptide sequence (39-42) of lactoferrin, has been studied on MMP-2 expression in synovial fibroblasts in the presence of nitric oxide donor \textit{in vitro}.

**MATERIALS AND METHODS**

**Chemicals**

Dulbecco’s Modified Eagle’s Medium (DMEM) and S-nitroso-N-acetyl-DL-penicillamine (SNAP) were obtained from M/s. Sigma Chem. Co., USA. Collagenase Type III was purchased from GIBCO BRL, Dimethyl sulfoxide (DMSO), RGDS and Anti-rat MMP-2 were sourced from Calbiochem Co.
Animals

Male *Wistar* rats weighing approximately 100 g, purchased from Tamil Nadu Veterinary and Animal Sciences University, Chennai, were used throughout this work. The rats were housed in solid bottomed polypropylene cages and fed with commercial rat diet (Hindustan Lever, Bombay, India) and water *ad libitum*. Animal care was as per Indian National Science Academy (INSA) Guidelines for the Care and Use of Animals in Scientific Research, and the study had an approval of Institutional Animal Ethical Committee (IAEC).

Induction of arthritis

Arthritis was induced in male Wistar rats as described earlier in Chapter II (Page No: 68). Arthritis was established on 14\textsuperscript{th} day after injection as confirmed by measurement of paw volume, histological and radiological studies. Animals were sacrificed on day 14 under deep anaesthesia and the ankle joints were dissected out for the preparation of synovial fibroblast culture.

Preparation of synovial fibroblasts from rats with CIA

Synovial fibroblasts were used from passage 3 to 6 for this study and they were cultured as described earlier in Chapter II, (Page No: 68).

Synthesis and characterization of peptides

PEP1261 and KRDS were synthesized and characterized as described earlier in Chapter II, (Page No: 70).
In vitro Studies

Cells were plated in 24-well or 48-well plates in Dulbecco’s modified Eagle's medium with 10% fetal bovine serum and cultured until 75-85% confluent. Cells were washed with Hanks' balanced salt solution three times and cultured for an additional 24 h in serum-free medium. Cells were stimulated by the addition of SNAP followed by the addition of peptides viz, PEP1261 (0.1mM), KRDS (1mM) and RGDS (1mM). The culture was terminated after 24 h and the supernatant was used for the estimation of nitrite level as well as for zymography and immunoblot. The cells were subjected to RT–PCR analysis for MMP-2 expression.

Nitrite assay

Nitrite levels were assayed by the method of Green et al (1982), as described in Chapter IV, ((Page No: 104).

Gelatin Zymography

Gelatinolytic activities were assessed under nonreducing conditions using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). 15 μl of supernatants mixed with 5 μl of loading buffer (0.16 M Tris-HCl, 50% glycerol, 8% SDS, and 0.08% bromophenol blue) were applied onto a 8% polyacrylamide gel copolymerized with 1 mg/ml gelatin. Electrophoresis was performed under constant voltage (150 V) for 3 h at 4°C. The gels were washed three times for 20 min each with 2.5% Triton X-100 (Sigma) to remove the SDS and to allow the electrophoresed enzymes to renature and they were incubated in zymography buffer (0.15M NaCl, 10 mM/L CaCl$_2$, 0.02% NaN$_3$ and 50 mM/L Tris-HCl, pH 7.5)
for 16 h at 37°C. The gels were then stained with 0.1% Coomassie brilliant blue G-250 in 2.5:1:7 ethanol:acetic acid:water and destained with 2:1:7 isopropanol:acetic acid:water.

**Western blotting**

SDS-PAGE electrophoresis followed by western blot were carried out as described earlier in Chapter III (Page No: 86).

**Preparation of RNA for mRNA studies**

Total cellular RNA was extracted from synovial fibroblasts using Trizol reagent, a mono-phasic solution of phenol and guanidine isothiocyanate developed by Chomczynski and Sacchi (1987).

Cells were lysed using 1ml of Trizol reagent. After addition of the reagent, the cell lysate was passed several times through a pipette to form a homogenous lysate. The homogenized samples were incubated for 5 min at 15°C to permit the complete dissociation of nucleoprotein complexes and 0.2 ml of chloroform per 1ml of Trizol reagent was added. Tubes were vigorously shaken by hand for 15 sec and incubated at 15°C for 3 min. The samples were centrifuged at 12,000 x g for 15 min at 4°C and following centrifugation, the mixture got separated into 3 phases: a red organic phase (containing protein), an interphase (containing DNA) and a colourless upper aqueous phase (containing RNA).

The aqueous phase was transferred to a fresh tube. 0.5 ml of isopropyl alcohol was used to precipitate the RNA from the aqueous phase. Samples were incubated at 15°C for 10 min and centrifuged at 12,000 x g for 15 min at 4°C. RNA
was pelleted with 75% ethanol. The sample was mixed by vortexing and centrifuged at 7,500 x g for 5 min at 4°C.

At the end of the procedure, RNA pellet was air dried for 10 min and the extracted RNA was quantified by spectrophotometry. The A<sub>260/280</sub> ratio of all preparations was greater than 1.8. To avoid contamination of the mRNA with genomic DNA, all samples were treated with 10 U of RNase-free DNase. RNA was redissolved in 10% formamide (deionised) and stored at -70°C until use.

**Specific MMP-2 primers and preparation of Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) products**

The first-strand cDNA was synthesized from the total RNA using Moloney Murine Leukemia Virus reverse transcriptase and oligo (DT) 18 primers (MBI Fermentas) according to the standard method. The reverse transcription reaction was performed at 42 °C for 1 h and at the end of reverse transcription, the mixture was heated at 95 °C for 5 min and immediately cooled on ice for 5 min. The second-strand DNA synthesis and 30-cycle amplification were then performed. Direct and reverse oligo primers for MMP-2 (5' GCT GAT ACT GAC ACT GGT ACT G 3' for sense and 5' CAA TCT TTT CTG GGA GCT C 3' for antisense) and β-actin (5' GGT ATG GGT CAG AAG GAC TCC 3' for sense and 5' TGA TCT TCA TGG TGC TAG GAG CC 3' for antisense) were constructed to amplify the full coding sequences according to the published sequences. Amplification was performed for 30 cycles of denaturation (60 s at 94 °C), annealing (60 s at 60 °C),
and elongation (90 s at 72 °C). PCR products were electrophoresed using 1.5 % agarose gel and visualized by ethidium bromide staining under UV light.

**Statistical Analysis**

Results were reported as mean ± standard deviation (S.D.). Statistical analysis was performed using one-way analysis of variance (ANOVA). If the overall $F$ value was found statistically significant ($p < 0.05$), further comparisons among groups were made according to post hoc Tukey's test. All statistical analyses were performed using SPSS statistical version 11 software package (SPSS Inc., USA).

**RESULTS**

Synovial fibroblasts were stimulated by the addition of SNAP (500µM) (Hirai *et al* 2001) for the production of nitric oxide and MMP-2 in synovial fibroblasts. Further studies were carried out to assess the inhibitory role of PEP1261(0.1mM), KRDS (1mM) and RGDS (0.1mM) on nitrite level and MMP-2 in SNAP treated cells.

Nitrite levels in the culture medium, which are direct indicators of the amount of nitric oxide produced, were found to be elevated in synovial fibroblasts treated with SNAP and this was significantly inhibited ($p<0.001$) upon treatment with PEP1261 (Fig 5.1).

As illustrated in Fig 5.2a, 72-kDa band with gelatinolytic activity was demonstrated in SNAP-treated synovial fibroblasts whereas SNAP with peptides treated synovial fibroblasts showed significant reduction in the gelatinolytic activity.
Fig 5.1: Effect of peptides on nitric oxide level in synovial fibroblasts. All values were mean ± S.D. (n=3). ## p<0.01 as compared to control and * p<0.05; **p<0.01 as compared to SNAP stimulated using non parametric Tukey’s post hoc test.
Fig 5.2a: Effect of peptides on gelatinase secretion in SNAP stimulated synovial fibroblasts. Lane1: Prestained molecular weight markers, Lane2: Control, Lane 3: S.Control, Lane 4: PEP1261, Lane 5: KRDS, Lane 6: RGDS, Lane 7: SNAP, Lane 8: SNAP + KRDS, Lane 9: SNAP + RGDS, Lane 10: SNAP + PEP1261

Fig 5.2b: Densitometric analysis of zymography of gelatinase secretion. All values were mean ± SD (n =3). ## p<0.01 as compared to control and * p<0.05; **p<0.01 as compared to SNAP stimulated using non parametric Tukey's post hoc test.
The relative densities of all gel bands were determined and the quantitative values were expressed as relative arbitrary units, with a significant decrease in the case of PEP1261 treated fibroblast cells (p<0.001) (Fig 5.2b).

Fig 5.3a showed typical immunoblotting analysis of MMP-2 in synovial fibroblasts from arthritic rats. There was a significant increase in MMP-2 production in the SNAP treated cells as compared to control group whereas SNAP and PEP1261 treated cells showed significant reduction in the MMP-2 production. Densitometric analysis also confirmed the significant increase in the MMP-2 production in SNAP treated synovial fibroblasts (Fig 5.3b). In addition, SNAP followed by PEP1261 treated cells showed significant reduction (p<0.001) in MMP-2 production.

Fig 5.4a elucidated the mRNA expression of MMP-2 and β-actin. MMP-2 mRNA was detected in SNAP stimulated synovial fibroblasts. In contrast, stimulation of these cells with SNAP followed by treatment with PEP1261 inhibited MMP-2 mRNA expression. There was no difference in the level of mRNA for β-actin, a control house keeping gene, between treated and untreated synovial fibroblasts. The relative densities of all gel bands were determined and quantitative values were expressed as relative arbitrary units, with a significant decrease in the case of PEP1261 treated synovial fibroblasts (p<0.001) when compared to KRDS and RGDS (Fig 5.4b).
Fig 5.3a: Western blot analysis of culture media. Effect of peptides on gelatinase secretion in SNAP stimulated synovial fibroblasts. Lane1; Prestained molecular weight markers, Lane2; Control, Lane 3; S.Control, Lane 4; SNAP, Lane 5; PEP1261, Lane 6; KRDS, Lane 7; RGDS, Lane 8; SNAP + PEP1261, Lane 9; SNAP + KRDS, Lane 10; SNAP + RGDS

Fig 5.3b: Densitometric analysis of western blot (n = 3). All values were mean ±SD (n =3). ## p<0.01 as compared to control and * p<0.05; **p<0.01 as compared to SNAP stimulated using non parametric Tukey's post hoc test.
Fig 5.4a: Effect of peptides on mRNA expression of MMP-2 in SNAP stimulated synovial fibroblasts. Total RNA was reverse transcribed following PCR amplification with primers for MMP-2 and b - actin. Lane1; Markers, Lane2; Control, Lane 3; S.Control, Lane 4; SNAP, Lane 5; PEP1261, Lane 6; KRDS, Lane 7; RGDS, Lane 8; SNAP + PEP1261, Lane 9; SNAP + KRDS, Lane 10; SNAP + RGDS

Fig 5.4b: Densitometric analysis of mRNA expression of MMP-2 (n = 3). All values were mean ± SD (n =3). ## p<0.01 as compared to control and * p<0.05; **p<0.01 as compared to SNAP stimulated using non parametric Tukey’s post hoc test.
DISCUSSION

Dysregulated metabolism of extracellular matrix, due to overproduction of MMPs might contribute to articular destruction observed in RA (Murphy et al., 2002). Activation of MMPs could occur through interaction with NO in the rheumatoid synovium. This study demonstrated the effects of exogenously generated NO on MMP-2 production from arthritic synovial cells. The data showed that a NO donor (SNAP) induced gelatinase B (MMP-2) production from synovial fibroblasts indicating that NO acted as a proinflammatory mediator in inflammatory joint diseases. Nitric oxide (NO) was a multifunctional mediator and it acted on various cells and participated in inflammation and autoimmune-mediated tissue destruction (Bredt and Snyder, 1994). NO levels were increased in synovial fluid in RA, and synoviocytes and chondrocytes were thought to be the primary producers of NO (McInnes et al., 1996; Amin et al., 1995).

Regulation of matrix metalloproteinase activity is crucial for the maintenance of a proper balance of tissue remodeling and injury (Werb and Chin, 1998). Recent studies suggested that a complex relationship existed between NO and MMPs. Reactive oxygen species, such as hydrogen peroxide, were potent and direct activators of MMP-2 (Belkhiri et al., 1997). In addition, Trachtman et al. (1996) demonstrated that stimulation of iNOS activity in rat mesangial cells by IFN-γ and LPS led to increased activity of 72 kDa matrix metalloproteinase. These findings suggested that NO might modulate the activity of 72 kDa MMP-2. ROS played an important role as signalling molecules as many cellular pathways were subjected to
redox regulation (Kamata and Hirata, 1999). MMPs were known to be activated by ROS (Belkhiri et al., 1997; Yoo et al., 2002) via various intracellular signalling pathways (Yoon et al., 2002). Thus, NO might induce MMP-2 production mediated through reactive oxygen molecules and ROS most probably played a central role in the SNAP-induced secretion of MMP-2 from synovial cells.

In this study, gelatin zymography clearly demonstrated that exogenously generated NO increased the gelatinolytic activity of MMP-2 derived from synovial fibroblasts from rat with arthritis. In addition, immunoblot analysis indicated NO induced MMP-2 production from synovial fibroblasts and RT-PCR analysis also demonstrated the induction of MMP-2 mRNA in SNAP-treated synovial cells. These results were in agreement with those reported earlier in rheumatoid synovial cells (Hirai et al., 2001). Cellular control of MMPs occurred at multiple levels including synthesis, secretion and activation/inhibition (Murphy et al., 1994). The mechanism by which NO modifies MMP-2 production is not clear at present.

The possible mechanism of the inhibition of MMP-2 secretion by PEP1261 could be related to its free radical scavenging properties as demonstrated, previously by the antioxidant properties of PEP1261 in adjuvant-induced arthritis in rats in vivo (Ashok Kumar et al., 2002). Earlier, it was noticed that PEP1261 inhibited ROS generation in neutrophils in AIA (Meera et al., 1999) and PEP1261 itself was a highly potent antioxidant (Ashok Kumar et al., 2002). Therefore, it is possible that PEP1261 exerted inhibition of MMP-2 secretion probably by inhibition of ROS.
In conclusion, the data presented herein demonstrated for the first time the MMP-2 inhibitory effects of PEP1261 in synovial fibroblasts from arthritic rats. Future studies will focus on deciphering the mechanism by which PEP1261 manifested its MMP-2 inhibitory effect.
SUMMARY

This study showed that SNAP (nitric oxide donor) enhanced MMP-2 expression in synovial fibroblasts and as such, it might be an important contributor to the development of the characteristic features of RA. In addition, this study demonstrated that nitric oxide upregulated MMP-2 mRNA expression in the fibroblasts, confirming the role of the nitric oxide in RA. Therefore, an attempt was made to examine whether PEP1261 was able to inhibit nitrite levels. Experiments performed with PEP1261 on synovial fibroblasts in vitro showed inhibition of nitrite level as well as MMP-2 mRNA expression.
REFERENCES


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