

Research article

# Collaborative interactions between neutrophil elastase and metalloproteinases in extracellular matrix degradation in three-dimensional collagen gels

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## Abstract

**Background:** Extended culture of monocytes and fibroblasts in three-dimensional collagen gels leads to degradation of the gels (see linked study in this issue, "Fibroblasts and monocytes contract and degrade three-dimensional collagen gels in extended co-culture"). The current study, therefore, was designed to evaluate production of matrix-degrading metalloproteinases by these cells in co-culture and to determine if neutrophil elastase could collaborate in the activation of these enzymes. Since co-cultures produce prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), the role of PGE<sub>2</sub> in this process was also evaluated.

**Methods:** Blood monocytes from healthy donors and human fetal lung fibroblasts were cast into type I collagen gels and maintained in floating cultures for three weeks. Matrix metalloproteinases (MMPs) were assessed by gelatin zymography (MMPs 2 and 9) and immunoblotting (MMPs 1 and 3). The role of PGE<sub>2</sub> was explored by direct quantification, and by the addition of exogenous indomethacin and/or PGE<sub>2</sub>.

**Results:** Gelatin zymography and immunoblots revealed that MMPs 1, 2, 3 and 9 were induced by co-cultures of fibroblasts and monocytes. Neutrophil elastase added to the medium resulted in marked conversion of latent MMPs to lower molecular weight forms consistent with active MMPs, and was associated with augmentation of both contraction and degradation ( $P < 0.01$ ). PGE<sub>2</sub> appeared to decrease both MMP production and activation.

**Conclusion:** The current study demonstrates that interactions between monocytes and fibroblasts can mediate tissue remodeling.

**Keywords:** collagen degradation, lung fibroblasts, metalloproteinases, monocytes, prostaglandin E<sub>2</sub>

## Introduction

Three-dimensional (3D) collagen gel culture has been used as an *in vitro* model of *in vivo* tissue contraction, a common feature of fibrosis, as well as the resolution of granulation tissue that characterizes repair [1,2]. Short-term co-cultures of monocytes with fibroblasts result in the

inhibition of collagen gel contraction [3], while co-cultures of fibroblasts with neutrophils, or with neutrophil elastase (NE), augment contraction [4].

Results in the linked study [5] demonstrated that 3D collagen gel contraction was augmented in extended co-cultures

of fibroblasts and monocytes. Since MMPs play a prominent role in connective tissue degradation [6–8], the current study, an extension of this linked study, was designed to explore the potential role of MMPs in this process.

## Materials and methods

See supplementary material for further information.

### Cells and cultures

See Supplementary material.

### Preparation of collagen gels for three-dimensional co-culture

Collagen gels were prepared as described previously [9]. For long-term co-culture, the medium was changed every 5 days. The areas of floating gels were measured using an image analyzer.

To investigate the effect of PGE<sub>2</sub> on collagen degradation, indomethacin (1 μM) or PGE<sub>2</sub> (0.1 μM) was added to the medium.

### Gelatinase activity assay

Gelatin zymography was performed by modification of a previously published procedure to identify MMPs 1 and -9 [10,11].

### Immunoblot analysis of metalloproteinases

To further identify the MMPs produced, immunoblots for MMPs 1 and 3 were performed.

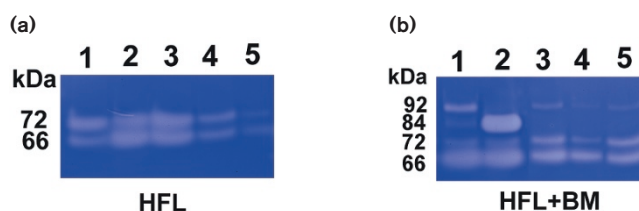
## Results

### Effect of co-culture on gelatinase activity

As shown in Figures 1 and 2, fibroblasts alone routinely released primarily MMP-2 into their surrounding medium, as identified at the molecular weights of 72 kDa (latent form) and 66 kDa (active form) (Fig. 1). Over 5 days, elastase appeared to partially convert some of the latent 72 kDa form to the 66 kDa form. With increasing incubation time, MMP-2 present in culture medium gradually decreased. Even at day 21, however, there was readily detectable MMP-2, consistent with ongoing release (Fig. 1a). Co-culture of monocytes and fibroblasts increased both bands of MMP-2 and resulted in more of the 66 kDa form (Fig. 1b). Co-culture of fibroblasts with monocytes also induced the release of MMP-9 (Fig. 1b), which was present as the latent 92 kDa form. Addition of elastase nearly completely converted the latent 92 kDa MMP-9 to the active 83 kDa form. With increasing culture time, the amount of detectable MMP-9 in co-cultures decreased. In contrast to the co-cultures, monocytes cultured alone released no gelatinolytic activity (data not shown).

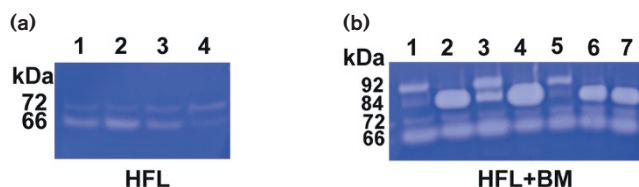
Neutrophil elastase (NE) augmented and PGE<sub>2</sub> inhibited the conversion of 72 kDa MMP-2 to the 66 kDa form in fibroblasts cultured alone (Fig. 2a). In co-cultures, indomethacin

**Figure 1**



Matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9). Culture media were harvested after 5, 10, 15 and 21 days under control conditions and, after 5 days, in the presence of neutrophil elastase (NE) and subjected to gelatin zymography. **(a)** Human fetal lung (HFL) fibroblasts alone. MMP-2 latent (72 kDa) and active (66 kDa) forms are shown. **(b)** Blood monocytes (BM) co-cultured with HFL fibroblasts. MMP-9 latent (92 kDa) and active (83 kDa) forms are shown in addition to MMP-2 forms. Lane 1: samples were harvested on day 5. Lane 2: culture medium in the presence of NE, harvested on day 5. Lanes 3, 4, 5: samples were harvested on days 10, 15 and 21 under control conditions, respectively.

**Figure 2**



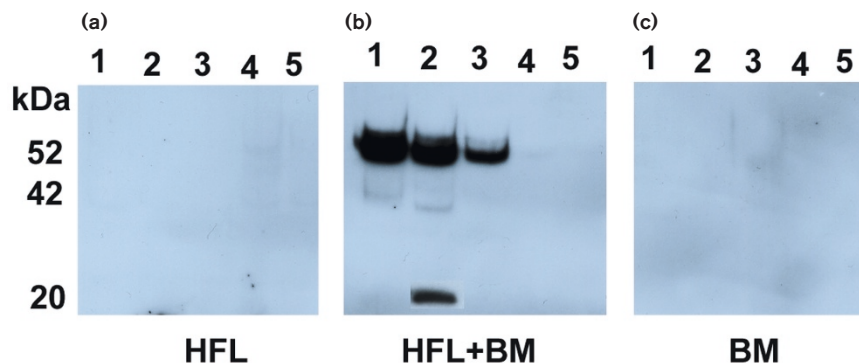
Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and matrix metalloproteinases (MMPs) 2 and 9. Culture media were harvested after four days in the presence of neutrophil elastase (NE), indomethacin or exogenous PGE<sub>2</sub>. **(a)** Fibroblasts alone. MMP-2 latent (72 kDa) and active (66 kDa) forms are shown. Lane 1: control; lane 2: NE; lane 3: indomethacin; lane 4: PGE<sub>2</sub>. **(b)** Monocytes co-cultured with fibroblasts. MMP-9 latent (92 kDa) and active (83 kDa) forms are shown in addition to MMP-2 forms. Lane 1: control; lane 2: NE; lane 3: indomethacin; lane 4: NE + indomethacin; lane 5: PGE<sub>2</sub>; lane 6: NE + PGE<sub>2</sub>; lane 7: NE + indomethacin + PGE<sub>2</sub>.

resulted in a marked increase of conversion of MMP-9 from the 92 kDa to the 83 kDa form, most readily observed in the absence of NE, where conversion was minimal (Fig. 2b). The addition of exogenous PGE<sub>2</sub> decreased the conversion of MMP-9 to the 83 kDa form MMP-9. Neither indomethacin nor PGE<sub>2</sub> induced release of gelatinase activity in monocytes cultured alone (data not shown).

### Effect of co-culture on MMP-1

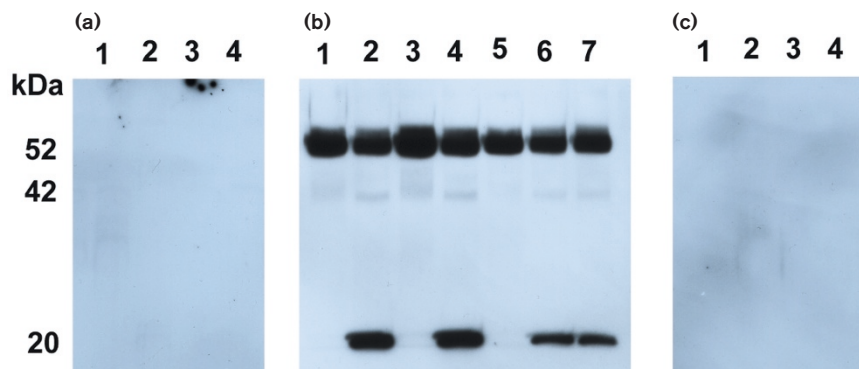
No detectable MMP-1 was observed in cultures of monocytes (Fig. 3). In fibroblasts alone, a trace of MMP-1 was occasionally detectable. In co-cultures of monocytes and fibroblasts, however, there was marked induction of MMP-1, which was present at a size corresponding to the latent 52 kDa form (Fig. 3). The detectable MMP-1 in co-cultures was maximal at earlier times, decreasing with increase cultured time and becoming undetectable by

Figure 3



Immunoblot for matrix metalloprotease (MMP)-1. Media were harvested after 5, 10, 15 and 21 days under control conditions and, after five days, in the presence of neutrophil elastase (NE) and were subjected to immunoblot for MMP-1. **(a)** Human fetal lung (HFL) fibroblasts alone. **(b)** Blood monocytes (BM) co-cultured with HFL fibroblasts. MMP-1 appears in its latent 52 kDa form. NE presence resulted in conversion of latent MMP-1 to active 42 kDa and 20 kDa forms. **(c)** BM alone. Lane 1: samples harvested on day 5. Lane 2: culture medium in the presence of NE, harvested at day 5. Lanes 3, 4, 5: samples harvested on days 10, 15 and 21 under control conditions, respectively.

Figure 4



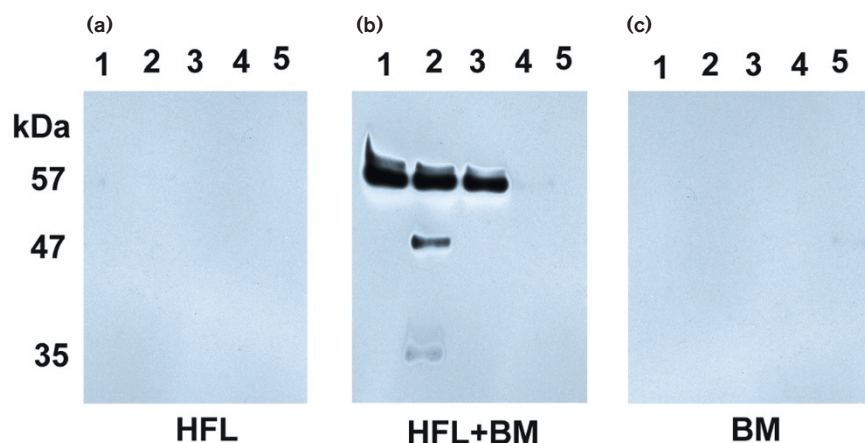
Effect of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) on matrix metalloprotease (MMP)-1. After four days, media were harvested for immunoblotting. **(a)** Human fetal lung (HFL) fibroblasts alone. Lane 1: control; lane 2: neutrophil elastase (NE); lane 3: indomethacin; Lane 4: PGE<sub>2</sub>. **(b)** Blood monocytes (BM) co-cultured with HFL fibroblasts. MMP-1 appears in its latent 52 kDa form. NE presence resulted in conversion of latent MMP-1 to the active 20 kDa form. Lane 1: control; lane 2: NE; lane 3: indomethacin; lane 4: NE + indomethacin; lane 5: PGE<sub>2</sub>; lane 6: NE + PGE<sub>2</sub>; lane 7: NE + indomethacin + PGE<sub>2</sub>. **(c)** BM alone. Lane 1: control; lane 2: NE; lane 3: indomethacin; lane 4: PGE<sub>2</sub>.

15 days (Fig. 3). The presence of neutrophil elastase for the first 5 days converted latent MMP-1 to active 42 kDa and 20 kDa forms. Indomethacin augmented the induction of MMP-1 in co-culture (Fig. 4).

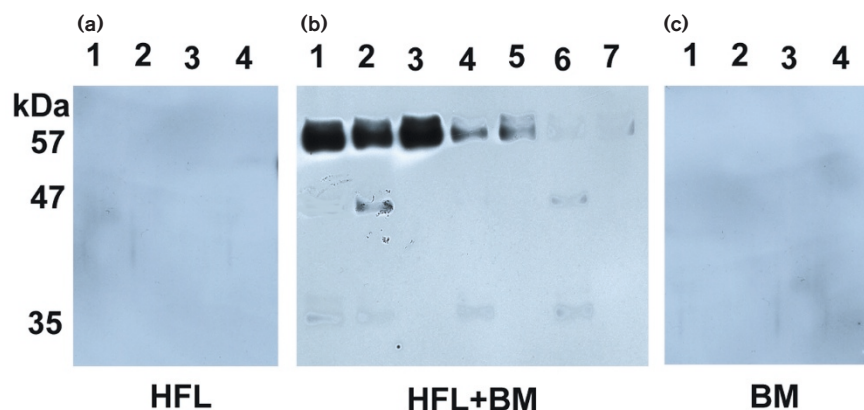
In contrast, PGE<sub>2</sub> reduced the amount of total MMP-1 and decreased the conversion of the 52 kDa form to the lower molecular weight forms in the presence of elastase. Neither indomethacin nor PGE<sub>2</sub> had an effect on MMP-1 in cultures of monocytes or fibroblasts alone.

**Effect of co-culture on MMP-3**

Neither fibroblasts nor monocytes alone released detectable MMP-3 (Fig. 5). In co-cultures of monocytes and fibroblasts, however, MMP-3 release was readily detected in a size corresponding to the latent 57 kDa form (Fig. 5). MMP-3 release was greatest at the earliest time points evaluated, and decreased with time becoming undetectable by 15 days of culture. Addition of NE for the first 5 days resulted in conversion of the 57 kDa form to active 47 and 35 kDa forms.

**Figure 5**

Immunoblot for matrix metalloprotease (MMP)-3. Media were harvested after 5, 10, 15 and 21 days under control conditions and, after five days, in the presence of neutrophil elastase (NE) and subjected to immunoblot for MMP-3. **(a)** Human fetal lung (HFL) fibroblasts alone. **(b)** Blood monocytes (BM) co-cultured with HFL fibroblasts. The latent 57 kDa form of MMP-3 is shown; addition of NE for the first five days resulted in conversion of the 57 kDa form to active 47 kDa and 35 kDa forms. Lane 1: samples were harvested on day 5. Lane 2: culture medium in the presence of NE, harvested at day 5. Lanes 3, 4, 5: samples harvested on days 10, 15 and 21 under control conditions, respectively. **(c)** BM alone.

**Figure 6**

Effect of prostaglandin  $E_2$  ( $PGE_2$ ) on matrix metalloprotease (MMP)-3. Media were harvested for immunoblotting. **(a)** Human fetal lung (HFL) fibroblasts alone. Lane 1: control; lane 2: neutrophil elastase (NE); lane 3: indomethacin; lane 4:  $PGE_2$ . **(b)** Blood monocytes (BM) co-cultured with HFL fibroblasts. Lane 1: control; lane 2: NE; lane 3: indomethacin; lane 4: NE + indomethacin; lane 5:  $PGE_2$ ; lane 6: NE +  $PGE_2$ ; lane 7: NE + indomethacin +  $PGE_2$ . **(c)** BM alone. Lane 1: control; lane 2: NE; lane 3: indomethacin; lane 4:  $PGE_2$ .

Indomethacin augmented the induction of MMP-3 while  $PGE_2$  reduced the conversion of MMP-3 to lower molecular weight forms (Fig. 6). Neither indomethacin nor  $PGE_2$  had an effect on MMP-3 on fibroblasts or monocytes cultured alone.

## Discussion

In pulmonary emphysema, various inflammatory mediators have been suggested to cause tissue destruction and loss of structure [12–15]. Several lines of evidence support the concept that neutrophil elastase contributes to the pathogenesis of emphysema [6,7]. Evidence, including the

marked expansion of macrophage numbers in smokers' lungs and in studies from genetically altered mice, also supports a role for macrophage-derived proteases in emphysema [16,17]. These concepts are not exclusive, and it is possible that several proteolytic and inflammatory mechanisms contribute to the development of emphysema.

In the linked study [5], extended co-cultures of fibroblasts and monocytes augmented collagen gel contraction and degraded the extracellular matrix. NE added to co-cultures resulted in a concentration-dependent degradation of collagen. The current study suggests that this increased degra-

duction of extracellular collagen may be due to NE activation of latent MMPs induced in the co-culture conditions.

NE has been demonstrated to result in the augmentation of contraction [4]. It also has been suggested to play an important role in the development of emphysema. In animals, instillation of NE can result in the development of pulmonary emphysema [18]. Individuals deficient in  $\alpha$ -1 protease inhibitor, moreover, have an increased susceptibility to the development of emphysema [19–21]. The current study suggests the possibility that NE can collaborate with MMPs, leading to the degradation of extracellular matrix.

The MMPs are a family of proteolytic enzymes [22,23]. Most are released as latent precursors. Proteolytic cleavage of the latent forms can result in generation of active proteases [24,25]. The MMPs differ both in their substrate specificity and in their mechanisms of activation. Since some members of the MMP family are capable of activating other members [26,27], it is likely that proteolytic cascades may regulate MMP activity. A further degree of regulation of MMP activity is afforded by the family of inhibitors: tissue inhibitors of metalloproteinases (TIMPs) [28].

Recent studies in genetically altered mice have suggested an important role for multiple proteases in the development of emphysema. Mice deficient in MMP-9, MMP-12, or NE are resistant to the development of emphysema or skin blisters [8,16]. Mice overexpressing collagenase, however, develop emphysema [14]. The current study proposes a possible collaboration among proteases that is responsible for the tissue degradation associated with the disease.

According to results from this study, several proteases are induced in co-cultures and activated in the presence of NE. It is likely that other proteolytic enzymes may also play a role beyond those evaluated in the current study. In this context, fibroblasts are known to express cell surface proteases, which may have a major role in regulating the activity of other mediators in the extracellular milieu [29–31]. It is of interest that PGE<sub>2</sub> appears to be able to regulate the protease activity responsible for extracellular matrix degradation.

It is unlikely that PGE<sub>2</sub> functions directly as a protease inhibitor. It seems more plausible that PGE<sub>2</sub> regulates proteolytic activity by altering the production of antiproteases, or by altering the production of components essential in the proteolytic cascade leading to collagen degradation [31]. The sequence of proteolytic events, by which NE leads to collagen degradation, is incompletely defined. PGE<sub>2</sub>, however, could potentially modulate the proteolytic cascade, resulting in collagen degradation at a number of steps. Both PGE<sub>2</sub> and a cascade of proteolytic events that lead to extracellular matrix degradation have the potential for serving as paracrine regulators. Such a means of regu-

lation may be particularly important in tissue remodeling. It seems unlikely that tissue remodeling is accomplished by individually active fibroblasts. Rather, coordinated activity within a tissue would seem to be a more appropriate means to accomplish alteration in tissue structure. Paracrine regulation would seem to be ideally suited to accomplish such an effect.

## Conclusion

This study demonstrates that monocytes and fibroblasts in co-culture can release MMPs and degrade extracellular matrix. Activation of MMPs by NE can augment this process. PGE<sub>2</sub> can modulate this proteolytic cascade. These data support a role for collaborative interaction among inflammatory mediators leading to tissue destruction in diseases such as emphysema.

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## Supplementary material

### Materials and methods

#### Cells and cultures

Human fetal lung fibroblasts (cell line HFL-1), obtained from the American Type Culture Collection (Rockville, MD,

USA), were cultured with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal-calf serum, 50 U/ml penicillin, 50 µg/ml streptomycin and 0.25 µg/ml fungizone. The cells were cultured in 100 mm tissue culture dishes (Falcon, Becton Dickinson Labware, Lincoln Park, NJ, USA). The fibroblasts were passaged every week. Subconfluent fibroblasts were trypsinized (trypsin-EDTA; 0.05% trypsin, 0.53 mM EDTA-4 Na) and used for collagen gel culture. Fibroblasts used in these experiments were between cell passages 14 and 16. Blood monocytes were isolated from blood cells of healthy blood donors [32]. Cell suspensions were >96% monocytes by the criteria of cell morphology on Wright stained cytosmeas. Monocytes were stored at 4°C and were used for co-culture within 4 hours after isolation.

#### Reagents

Human neutrophil elastase was purchased from ECP (Owensville, MO, USA). Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and indomethacin were purchased from Sigma (St. Louis, MO, USA). Tissue culture supplements and media were purchased from GIBCO (Life Technologies, Grand Island, NY, USA). Fetal calf serum was purchased from Biofluid (Rockville, MD, USA).

#### Preparation of collagen gels

Tendons were excised from rat tails, and the tendon sheath and other connective tissue were removed carefully. After repeated washing with Tris-buffered saline (TBS, 0.9% NaCl, 10 mM Tris, pH 7.5) and serial concentrations of ethanol (from 50% to 100%), type I collagen was extracted in 6 mM hydrochloric acid at 4°C for 24 hours. Protein concentration was determined by weighing a lyophilized aliquot from each lot of collagen solution. Sodium dodecyl sulfate polyacrylamide gel electrophoresis routinely determined no detectable protein other than type I collagen.

#### Gelatinase activity assay

Conditioned media were concentrated fivefold by ethanol precipitation and re-suspension in distilled H<sub>2</sub>O. The samples were dissolved in twofold electrophoresis sample buffer (0.5 M Tris-HCL, pH 6.8, 2% SDS, 20% glycerol, 0.1% bromophenol blue), and heated for 5 min at 95°C. Thirty microliters of each sample were loaded in each lane, and electrophoresis was performed with a Mini Electrophoresis Cell (BIO-RID, Hercules, CA, USA) at 200 V. After electrophoresis, the gels were gently soaked with 2.5% (v/v) Triton-X 100 at 20°C for 30 min, then incubated in the metalloproteinase buffer (0.06 M Tris-HCl, pH 7.5, containing 6 mM CaCl<sub>2</sub> and 1 µM ZnCl) for 18 hours at 37°C. The gels were stained with 0.4% (w/v) Coomassie blue and rapidly destained with 30% (v/v) methanol, 10% acetic acid. The gels were dried directly between cellophane sheets (Pharmacia Biotech, San Francisco, CA, USA).

### **Immunoblot analysis of metalloproteinases**

Supernatant media from 3D cultures were concentrated 10-fold by precipitation with ethanol, resuspended in distilled H<sub>2</sub>O and mixed with twofold sample buffer (0.5 M Tris-HCl, pH 6.8, 2% SDS, 0.1% bromphenol blue, 0.5% β-mercaptoethanol, 20% glycerol). After heating for 3 min at 95°C, 30 μl of each sample was loaded for electrophoresis with a Mini Electrophoresis Cell (BIO-RAD, Hercules, CA). The proteins were transferred to a PVDF transfer membrane (BIO-RAD, Hercules, CA, USA) in electrophoresis buffer (20 mM Tris-HCl, pH 8.0, 150 mM glycine, 20% methanol) at 20 V for 35 min with a Semi-dry Electrophoretic Transfer Cell (BIO-RAD, Hercules, CA, USA). The blots were blocked in 5% fat-free milk in PBS-Tween at room temperature for 1 hour, then exposed to primary antibodies (mouse anti-human MMP-1, MMP-2, MMP-3 or MMP-9 antibodies; Calbiochem, Cambridge, MA, USA), and subsequently detected using HRP conjugated rabbit anti-mouse IgG (ICN Biomedical, Costa Mesa, CA, USA) in conjunction with an enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England).