The −564 A/G polymorphism in the promoter region of the proteinase 3 gene associated with Wegener's granulomatosis does not increase the promoter activity

K. PIETERS*, Å. PETTERSSON†, U. GULLBERG* & T. HELLMARK† *Department of Haematology, Lund University, Lund, Sweden, and †Department of Nephrology, Lund University, Lund, Sweden

SUMMARY

Proteinase 3 is the major autoantigen in patients with Wegener's granulomatosis. Earlier studies have shown that circulating leucocytes from patients with Wegener's granulomatosis show elevated proteinase 3 surface expression and mRNA levels. Wegener's granulomatosis patients also have increased levels of proteinase 3 in plasma. A single nucleotide polymorphism (SNP) (− 564 A/G SNP) in the promoter region has been associated with disease. This SNP introduces a new potential Sp1 transcription factor binding site that may be responsible for the observed up-regulated expression of proteinase 3. To investigate this a 740 base pair long region of the promoter was cloned from genomic DNA. The disease-associated −564 A/G, as well as a control −621 A/G exchange, were introduced by polymerase chain reaction mutagenesis and cloned into a luciferase reporter vector. Endogenous expression levels of proteinase 3 mRNA and promoter activity of the cloned constructs were measured in three myeloid cell lines, HL-60, U937 and NB-4, and in epithelial HeLa cells. The results demonstrate a good correlation between the endogenous proteinase 3 mRNA expression and the promoter activity, as judged by luciferase activity. However, no significant differences in activity between the wild-type, polymorphic and the mutated control variant were found. In conclusion, the −564 A/G polymorphism is not responsible for the increased expression levels seen in myeloid cells from patients with Wegener's granulomatosis.

Keywords   ANCA   promoter, proteinase 3   vasculitis   Wegener's granulomatosis

INTRODUCTION

Wegener's granulomatosis and microscopic polyangiitis are systemic small vessel vasculitides of unknown aetiology. Both diseases are associated with autoantibodies against granule proteins from neutrophils and monocytes, e.g. proteinase 3 and myeloperoxidase [1]. These antibodies are called antineutrophil cytoplasmic antibodies (ANCA). Autoantibodies against proteinase 3 are found in about 85% of patients with Wegener's granulomatosis and in 45% of patients with microscopic polyangiitis [2,3]. Some correlations are found between antibody titres and clinical activity, but the mechanism by which they are formed is unclear and it is still an unresolved issue whether the ANCA are pathogenic in humans.

More recent studies have shown that proteinase 3 itself plays an important role in the disease process. The enzyme is expressed on the surface of a subset of neutrophils [4–6]. A high percentage

of neutrophils expressing proteinase 3 on their surface is considered a risk factor for disease progression in Wegener's granulomatosis patients [6,7]. Primed neutrophils can be fully activated by ANCA, probably via cross-linking of the surface-bound proteinase 3 and the Fc receptor [8,9]. Moreover, the finding of increased expression of the protein in disease provides indirect evidence for a pathological role of proteinase 3. We and others have found elevated levels of circulating proteinase 3 in plasma from patients with Wegener's granulomatosis [10–12]. Furthermore, mRNA levels of proteinase 3 are also raised in monocytes of patients with Wegener's granulomatosis [13]. The molecular mechanism for increased expression of proteinase 3 in vasculitides is not clear, but possible mechanisms include increased transcription of the proteinase 3 gene.

Several transcriptional control elements have been defined in the promoter region upstream of exon 1 of the proteinase 3 gene. Within the first 200 base pairs of the proteinase 3 promoter, Sturrock *et al.* identified two elements, a PU.1 binding site at position −101 and a cytidine-rich site at position −190. Both elements are critical for maximal activity of the analysed promoter. The cytidine-rich site shows sequence similarities to binding sites of the transcription factor Sp1, but direct binding of Sp1 could not be

Correspondence: Katrien Pieters, Department of Haematology, BMC-C14, SE-22184 Lund, Sweden.

E-mail: Katrien.Pieters@hematologi.lu.se

demonstrated [14]. PU.1 nuclear factor did bind directly to the PU.1 site and this binding was reduced during myeloid differentiation in correlation with a reduced proteinase 3 mRNA expression [14]. Another study showed that up-regulation of proteinase 3 mRNA expression by G-CSF is associated with PU.1 binding [15], lending further support for PU.1 as an important transcription factor for proteinase 3. The activity of the promoter of proteinase 3 is also dependent on the presence of sites for C/EBP and c-Myb and on a TATA site within the first 91 base pairs of the promoter [16].

In 1999, Gencik *et al.* reported an A→G SNP in the promoter region of proteinase 3 that give rise to a new potential Sp1 transcription factor binding site 564 base pairs upstream from the translation start [17]. Furthermore, the G allele frequency was significantly higher in patients with Wegener's granulomatosis compared to healthy individuals. Considering that a similar SNP is present in the myeloperoxidase gene [18] and that this SNP confers a 25-fold increase in expression [19], we speculate that the −564 A/G SNP in the proteinase 3 promoter region could be responsible for the increased expression of proteinase 3 found in patients with ANCA associated small vessel vasculitis. Therefore, we wanted to investigate the role of the Sp1 site created in the G allele in terms of increased promoter activity.

MATERIALS AND METHODS

Cloning of DNA and sequence analysis

The promoter region of the proteinase 3 gene was obtained by polymerase chain reaction (PCR) amplification of genomic DNA collected from mononuclear blood cells. In the first PCR an amplicon was obtained by running a PCR with a Pfu polymerase (Stratagene, La Jolla, CA, USA) using the following primers: 5′-CAGTGGCAC GATCTTGGCT-3′ and 5′-GCTCACTCACCGC TCAGCA-3′. The PCR product was amplified further in a second

PCR using two nested primers: 5′-GGTA<u>CTCGAG</u>CAAA TAATGAA CACTGGTCTCTCCC-3′ and 5′-ACTG<u>AAGCTT</u> GGTGGGGT CCAGGGTGC-3′. These primers also introduced the *Xho*I and *Hin*dIII restriction sites (underlined) for subsequent cloning of the second PCR product. The PCR product and the pGL3 basic vector (Promega) were cleaved, purified and ligated. The construct was sequenced and found to have an A in the −564 position. This construct was defined as proteinase 3 wild-type.

Mutagenesis

A sequence analysis revealed a second putative site, 57 base pairs further upstream of the −564 G/A SNP, in which a single mutation from A to G will introduce another Sp1 site (− 621 A/G). This site is not reported as a naturally occurring SNP in humans, but was used in the study as a control. Both the previously described SNP −564 A/G and the −621 A/G (control) site were created by mutating an A→G by site directed mutagenesis using the primers 5′-GGACCCTGGGCGAGGTCTGAG-3′ and 5′-ATGGAGTG GGCGGAGGCCAAG-3′, respectively, and the megaprimer method [20]. The PCR products were purified and ligated into the pGL3 basic vector and sequenced. The two constructs were called −564 A/G and −621 A/G, respectively, (Fig. 1).

Cell culture

U937 (ATCC no CRL-1593·2), NB-4 (DSMZ no. ACC 207) and HeLa (ATCC no. CCL-2) cells were cultured in RPMI-1640 (Gibco, BRL) supplemented with 10% fetal calf serum (Gibco BRL Invitrogen AB, Sweden). HL60 clone 15 (ATCC no. CRL-1964) was cultured in identical medium supplemented with 1 mM L-glutamine and 1 mM sodium pyruvate. U937, NB-4 and HL60 clone 15 are human myeloid leukaemia cell lines expressing endogenous proteinase 3. HeLa is a human cervix carcinoma cell line that is negative for proteinase 3.
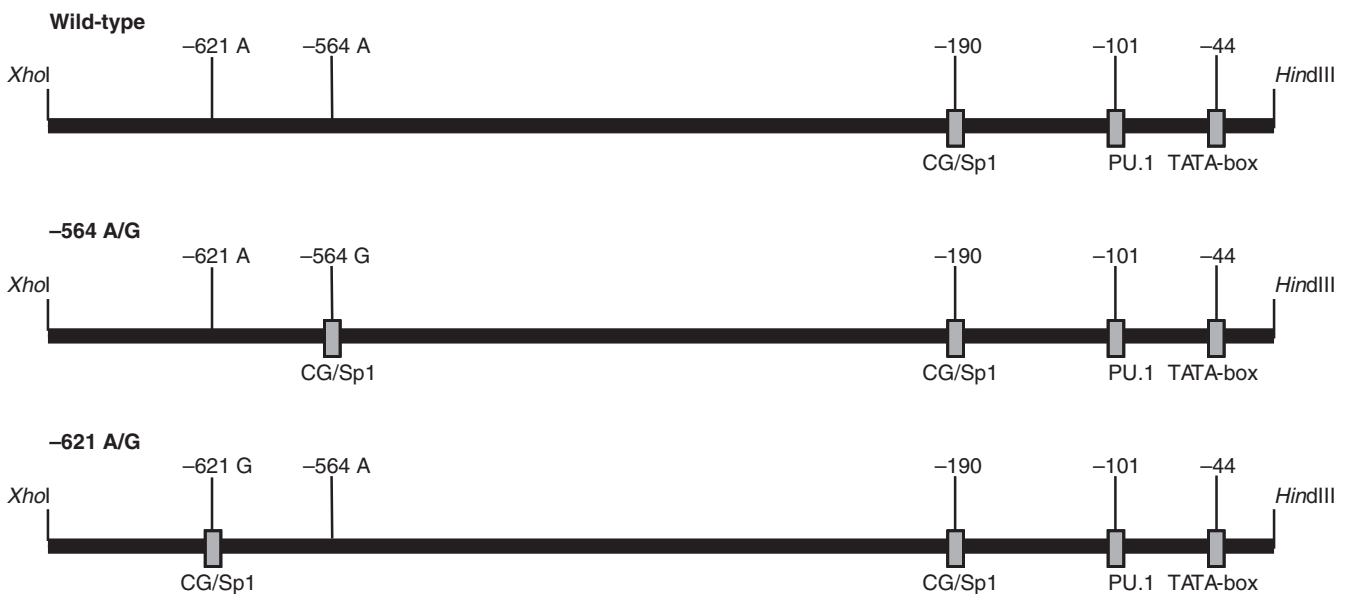
Fig. 1. A schematic drawing of the three different proteinase 3 promoter constructs. The wild-type vector has no additional Sp1 sites to the putative one in the CG rich element. The −564 A/G construct carries the reported SNP [17] introducing a new Sp1 site at position −564. The −621 A/G construct is mutated at position −621 from an A→G also yielding a Sp1 site. The −621 A/G SNP is not reported to occur in humans and this construct serves as a control only.

Total RNA extraction

Exponentially growing U937, NB-4, HeLa and HL60 cells were harvested and total RNA was isolated using the Trizol LS reagent kit (Invitrogen, Life Technologies, Sweden) according to the manufacturer's instructions. RNA-concentration was determined from A260 using the Nanodrop® ND-1000 Spectrophotometer (Nanodrop Technologies, Delaware, USA).

Real-time reverse transcription-polymerase chain reaction (real time RT-PCR)

Fifty ng of RNA converted to cDNA, using oligo $d(T)_{16}$ primers and the *Taq*Man® Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA), was used per 25 μl reaction in which proteinase 3 cDNA was amplified by quantitative real-time PCR (*Taq*Man®) using an ABI PRISM 7000 Sequence Detector (Applied Biosystems) according to the manufacturer's instructions. Primers and probe were purchased from Applied Biosystems (Assays-on-Demand no. HS-00160521_m1). RT-real time PCR of β2-microglobulin (Applied Biosystems no. 4326319E) was used as internal control for equal loading. Data were collected and analysed with Sequence Detector version 1·1 software (Applied Biosystems). Relative quantitative data were calculated based on the $\Delta\Delta C_T$ method: normalization: ($\Delta C_T = C_T$ (sample) − C_T (β2-microglobulin); $\Delta\Delta C_T = \Delta C_T$ (sample) − ΔC_T (calibrator); relative quantification = $2^{-\Delta\Delta CT}$ [21].

Transient transfections and luciferase assay

Conditions for electroporation were optimized for each cell line to obtain maximal luciferase activity, as described previously [22]. U937, NB-4 and HeLa cells were transiently transfected with 15 μg of pGL3-reporter plasmid and 0·1 μg *Renilla* vector, pRL-SV40 (Promega) as internal control and HL60 cells with 35 μg reporter plasmid and 0·1 μg *Renilla* vector. Electroporation was performed in a 0·4 cm cuvette (Bio-Rad Hercules, CA, USA) with cells suspended in 500 μl culture medium using Genepulser II equipment (Bio-Rad). Cell number and electrical settings during electroporation were as follows: U937: 8×10^6/ml, 280 V, 960 μF; NB-4: 8×10^6/ml, 280 V, 960 μF; HL60: 5×10^6/ml 340 V, 960 μF. HeLa cells were plated at a density of 2×10^6/well in a six-well culture plate and transfected the next day at 70% confluence using the Lipofectin® reagent (Invitrogen, Life Technologies). After transfection, U937 cells were incubated for 16 h, NB-4 and HL60 cells for 10 h and Hela-cells for 20 h, after which the amount of luciferase was determined using a Dual-Luciferase reporter kit (Promega SDS Biosciences, Sweden) according to the manufacturer's instructions and a TD-20/20 luminometer (Turner Design Sunnyvale, CA, USA). Firefly luciferase values were corrected for the transfection efficiency by normalization to the *Renilla* luciferase values.

RESULTS

Proteinase 3 mRNA expression in myeloid cell lines

For our studies we chose the monocytic cell line U937 and the promyelocytic cell lines NB-4 and HL60, all expressing endogenous proteinase 3 mRNA [23–25]. As a negative control we used the epithelial HeLa cell line without proteinase 3 expression [14]. In order to determine the relative amount of endogenous proteinase 3 mRNA in the various tested cell lines we performed real-time RT-PCR analyses. In all the myeloid cell lines, U937, NB-4 and HL60 proteinase 3 mRNA expression was verified. The

Table 1. Expression levels of proteinase-3 mRNA in different cell lines. Relative expression of mRNA was determined by *Taq*Man real-time RT-PCR. Shown are mean values of two independent experiments performed in duplicate and normalized to those of U937 cells

Cell line	Relative expression
U937	1·0
NB-4	13·0
HL60	480·5
HeLa	Undetectable
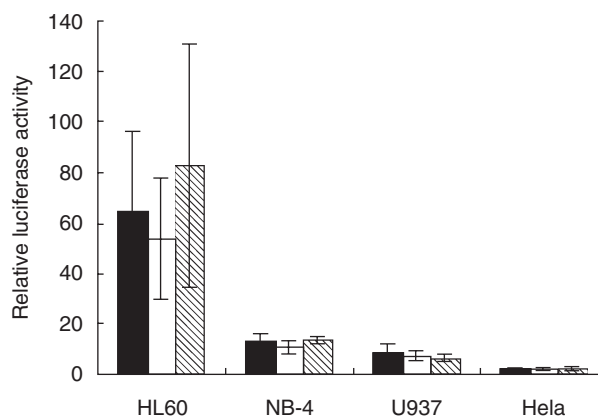
Fig. 2. Proteinase 3 promoter activities in different cell lines. Variant wild-type (filled bars), variant −564 A/G (open bars), and variant −621 A/G (marked bars) of the proteinase 3-promoter were transiently transfected into HL60, NB-4, U937 and HeLa cells, respectively. Luciferase activity was determined as described in Materials and methods. Values shown are normalized to those obtained by transfection with pGL3 basic vector. Shown are mean ± s.e.m. values from three independent experiments, each one performed in duplicate.

weakest expression level of proteinase 3 mRNA was present in U937 cells, while NB-4 and HL60 showed intermediary and high levels, respectively (Table 1). In HeLa cells the proteinase 3 mRNA expression was undetectable, as expected.

Promoter activity in myeloid cell lines

In order to reveal possible differences in transcriptional activity between the three polymorphic variants of the proteinase 3 promoter (wild-type, −564 A/G and −621 A/G), we transfected promoter-reporter plasmids to the cell lines. As shown in Fig. 2, three variants of the proteinase 3 promoter showed the highest luciferase activity in HL60 cells, compared to the activity in the two other myeloid cell lines NB-4 and U937. When transfected to non-myeloid HeLa-cells no significant promoter activity was seen. Thus, the promoter activity shows good correlation to the levels of expression of endogenous proteinase 3 (Table 1) and demonstrates that the promoter confers a myeloid-specific expression. Within each cell line, however, none of the three different promoter fragments showed any significant difference in activity (Fig. 2).

In conclusion, the high expression level of proteinase 3 mRNA in HL60 cells is in good correlation with the high promoter activity in this cell line. However, the three different promoter variants showed no significant mutual differences in

activity in any of the cell lines analysed, arguing strongly against the polymorphic variants conferring different expression levels of proteinase 3 mRNA.

DISCUSSION

Understanding the transcriptional, and perhaps the post-transcriptional, regulation of proteinase 3 expression is fundamental to understand the quantitative differences in the expression of proteinase 3 in patients with Wegener's granulomatosis and healthy people [6,12,26]. Certain identified polymorphisms in the proteinase 3 gene and promoter [17] were found to be over-represented in patients with Wegener's granulomatosis, but whether or not the polymorphisms are indeed causing quantitative differences in proteinase 3 expression has so far not been revealed. Our hypothesis in this study was that the previously identified proteinase 3 promoter polymorphism, an A→G SNP at position –564, results in increased proteinase 3 promoter activity contributing to increased proteinase 3 expression.

To test this hypothesis, we transiently transfected promyelocytic HL60 and NB-4 cells and monocytic U937 cells, and HeLa with three variants of the proteinase 3 promoter (–564 A/G; –621 A/G and wild-type) (Fig. 1). While HL60 and NB-4 are of promyelocytic phenotype, U937 cells show some monocytic characteristics. The highest promoter activity was found in HL60 cells, which also was the cell line showing highest levels of endogenous proteinase 3 mRNA, indicating that analysed promoter activity correlates to endogenous gene expression. However, within each cell line, our data revealed no significant differences in promoter activity among the three different promoter fragments, thus arguing strongly against our hypothesis.

Another common target antigen of ANCA in small-vessel vasculitis is myeloperoxidase which is, like proteinase 3, expressed at the promyelocytic stage during neutrophil maturation and shows a similar expression pattern with proteinase 3 during normal myeloid differentiation [27]. One study [18] showed an association between a common and functional myeloperoxidase promoter polymorphism (–463 G/A) and an increased incidence of ANCA-associated small-vessel vasculitis. This association could not, however, be confirmed by Fiebeler *et al.* [28]. Another study revealed that this common polymorphism (–463 G/A) in the myeloperoxidase promoter created a functional Sp1 binding site resulting in a 25-fold increase of transcriptional activity [29].

Given that the –564 A/G SNP of the proteinase 3 promoter also creates a novel potential Sp1 binding site, we expected an increased transcription from this SNP variant. This was not found to be the case, explained possibly by the long distance between the new/additional potential binding site for Sp1 and the TATA-box. A study on the effect on transcription of two GC sites (i.e. an additional Sp1 transcription binding site) at various distances from the TATA box in the E1B promoter showed the importance of the extremely close spacing between the GC site and the TATA box [30]. The previously reported GC-rich potential Sp1 site in the wild-type proteinase 3 promoter is localized at –190 base pairs up-stream from the proteinase 3 gene and is separated from the TATA box by only 146 base pairs (Fig. 1) [14,31], consistent with the importance for maximal activity of the promoter. However, the additional potential Sp1 binding site at position –564, created by the –564 A/G SNP, is 520 base pairs separated from the TATA box, which might be too far to positively affect the transcriptional activity of the promoter. The reason why the

additional Sp1 site at position –463 in the myeloperoxidase promoter gives an increased promoter activity, and not the additional Sp1 site at position –564 in the proteinase 3 promoter, might be the close distance of the Sp1 site to the alternative transcriptional start site P2 in the myeloperoxidase promoter [32]. In summary, our results argue strongly against the hypothesis that the –564 A/G SNP in the promoter region of proteinase 3 is increasing the promoter activity and thus being the mechanism explaining the elevated levels of proteinase 3 found in patients with Wegener's granulomatosis.

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