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Novel autoantigens of perinuclear anti-neutrophil cytoplasmic antibodies (P-ANCA) in ulcerative colitis: non-histone chromosomal proteins, HMG1 and HMG2

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SUMMARY

Anti-neutrophil cytoplasmic antibodies (ANCA) in sera from ulcerative colitis (UC) patients have been described as reacting with proteins in the granules of human neutrophils such as cathepsin G and lactoferrin and with yet unidentified antigens. Here we report the existence of a new member of perinuclear ANCA (P-ANCA) in UC patients. In the previous study, we found that UC patients had a novel P-ANCA against neutrophil 28-kD protein. In this study, we purified the same antigens from HL-60 lysates by using reversed phase high-performance liquid chromatography, and revealed that the 28-kD antigen consisted of two different proteins. The N-terminus amino acids of these proteins are identical with those of high mobility group (HMG) non-histone chromosomal proteins HMG1 and HMG2. Immunoblotting analysis of human neutrophil lysates using rabbit anti-HMG1/2 antisera revealed a single band of 28 kD, and the 28-kD band detected by immunoblotting analysis using patient's serum IgG completely disappeared after preincubation with a mixture of HMG1 and HMG2. Furthermore, rabbit anti-HMG1/2 antisera showed a perinuclear staining pattern in indirect immunofluorescence studies using ethanol-fixed neutrophils. These data demonstrate that HMG1 and HMG2 are novel target antigens of P-ANCA. HMG1 and HMG2 are distributed in the nuclei and cytoplasm of eukaryotic cells and act as transcription factors. Their intracellular localization and functions are distinct from those of the previously reported granular antigens of P-ANCA.

Keywords ANCA HMG1 and HMG2

INTRODUCTION

ANCA is divided into the cytoplasmic (C-ANCA) and the perinuclear (P-ANCA) type by indirect immunofluorescence (IIF) [1]. P-ANCA pattern is detected in 30–83% of sera from patients with ulcerative colitis (UC) [2–6], and some of the antigens associated with P-ANCA have been shown to be lactoferrin and cathepsin G [7,8]. In our previous study, we tested ANCA from sera of 35 UC patients [9]. Twenty-two sera were positive for P-ANCA by IIF. Among them, seven sera showed a 28-kD band in immunoblotting analysis of human neutrophil lysates. Five of these seven patients suffered from refractory UC. Thus, it appeared that an antibody against neutrophil 28-kD antigen serves as a predictive marker of refractory UC. Although the molecular size of the 28-kD protein was similar to that of cathepsin G, we proved that they were distinct from each other [9]. Here, we report the purification and identification of this protein.

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MATERIALS AND METHODS

Materials

Neutrophils were isolated from peripheral blood obtained from normal human volunteers by Ficoll density gradient centrifugation. The human promyelocytic leukaemia cell line HL-60 (ATCC CCL 240) was obtained from ATCC (Rockville, MD). Porcine high mobility group (HMG) 1 and HMG2 were purified from pig thymus as described elsewhere [10]. Their purity was confirmed by SDS–PAGE. Antisera to porcine HMG1 and HMG2 were elicited in rabbits. Briefly, male New Zealand White rabbits were injected subcutaneously with 20 mg porcine HMG1 or HMG2 emulsified 1:1 with Freund's complete adjuvant (FCA; Sigma, St Louis, MO). The rabbits were injected intravenously with the same antigen in PBS twice before confirming the antibody activity by immunoblotting. The mixture of bovine HMG1/HMG2 was purchased from Wako (Osaka, Japan). The bovine HMG1/HMG2 mixture showed two bands of 29 kD and 28 kD in SDS–PAGE.

Patients

Sera from seven patients with UC and whose serum contained an antibody against neutrophil 28-kD antigen in immunoblotting

analysis were studied. Five of them suffered from refractory type UC. The refractory type was defined according to the previous report [9].

Protein electrophoresis and immunoblotting

SDS–PAGE was performed as described by Laemmli [11]. Briefly, samples were boiled in SDS sample buffer (2% SDS, 50 mM Tris–HCl pH 6·8, 5% 2-mercaptoethanol, 5% glycerol and 0·002% bromophenol blue) for 10 min at 85°C before electrophoresis on 3% stacking and 10% running polyacrylamide gels. For immunoblotting, the samples were transferred to Immobilon sheets (Millipore, Bedford, MA). The sheets were then blocked with 5% skim milk for 1·5 h at room temperature, and incubated with antibodies overnight at 4°C. The secondary antibodies used were horseradish peroxidase-conjugated antibodies specific for either rabbit IgG (Zymed, San Francisco, CA) or human IgG (Cappel, Durham, NC). The reaction was visualized by incubation with ECL detection reagents (Amersham, Aylesbury, UK).

Purification of the 28-kD protein

Fractions containing the 28-kD protein were identified by immunoblotting of the samples with protein A-purified IgG from five patients who had antibodies to neutrophil 28-kD protein. As samples, HL-60 cells (2×10^9) were treated with 6 M guanidine HCl followed by sonication. After centrifugation at 80 000 ***g*** for 30 min, the supernatant was dialysed against PBS. The dialysate was collected to fractionate the 28-kD/29-kD antigens by reversed phase high-performance liquid chromatography (HPLC) on a YMC protein RP column (250 × 4·6 mm). The column was pre-equilibrated in 0·1% trifluoroacetic acid (TFA) and the protein eluted with a linear gradient of 16–48% acetonitrile containing 0·1% TFA/30 min at 1·5 ml/min. Fractions were monitored by absorbance at 214 nm and were dried under vacuum. The residues were dissolved in SDS sample buffer and analysed by SDS–PAGE and immunoblotting, which revealed that the peaks at 18 min and 22 min corresponded to 28 kD and 29 kD, respectively. These procedures were repeated twice.

Protein sequencing

The fractions of the 28-kD/29-kD antigens of interest were finally transferred onto ProBlott membrane (Applied Biosystems, CA). Protein bands were stained with Ponceau S (Sigma, St Louis, MO) after electroblotting and positive bands were cut out. Automated Edman degradation was then carried out on the blotted proteins using PPSQ-10 (Shimadzu, Kyoto, Japan), which revealed the sequence of the N-terminal amino acids.

IIF assay

ANCA activity was confirmed by IIF as reported by Wiik [12]. Briefly, ethanol-fixed cytospin preparations of human neutrophils were incubated with rabbit antiserum against porcine HMG1/2 at room temperature for 1 h. As a control, normal rabbit IgG (Dako, Glostrup, Denmark) was employed. As secondary antibody, FITC-conjugated anti-rabbit IgG (Cedarlane, Ontario, Canada) was used. The slides were evaluated by fluorescence microscopy. Samples positive for perinuclear staining were subjected to formalin fixation, instead of ethanol fixation, to distinguish ANCA from antinuclear antibodies [13].

RESULTS

Purification and sequencing of the 28-kD protein

We selected sera of five patients with UC which exhibited a 28-kD band in immunoblotting of human neutrophil lysates. The mixture of IgG obtained from these sera also exhibited a similar band in immunoblotting using lysates of the human promyelocytic leukaemia cell line HL-60, as shown in Fig. 1. We used HL-60 cells as the source for purification in subsequent experiments.

The cell lysate of HL-60 was fractionated by reversed phase HPLC, as shown in Fig. 2a. The two fractions, indicated by arrows in Fig. 2a, reacted with protein A-purified IgG from the five patients who had antibodies to neutrophil 28-kD protein. The two fractions were further purified by SDS–PAGE and transferred to the ProBlott membrane. SDS–PAGE and immunoblotting

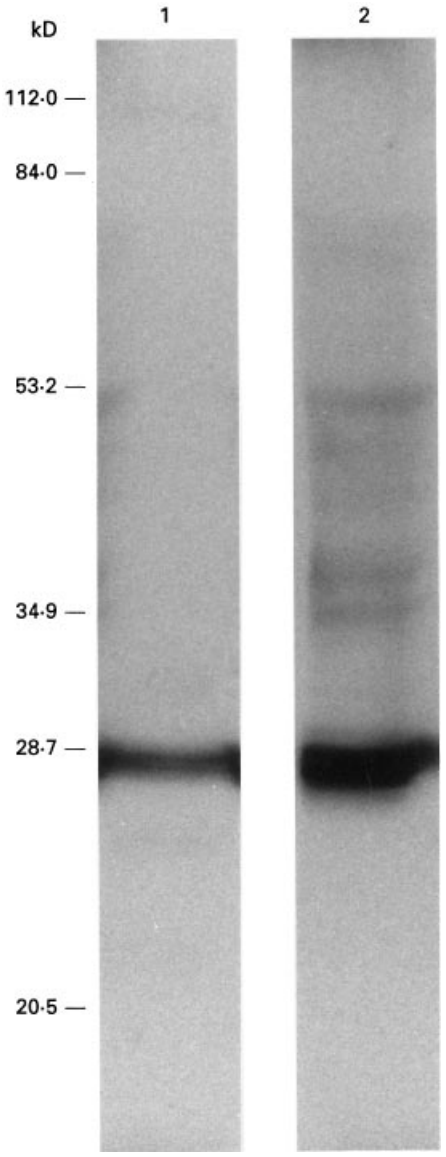

Fig. 1. Analysis of the 28-kD antigen from HL-60 lysates by immunoblotting. Immunoblotting demonstrated that the 28-kD neutrophil antigen (lane 1) has the same molecular weight as the corresponding antigen from HL-60 lysates (lane 2).

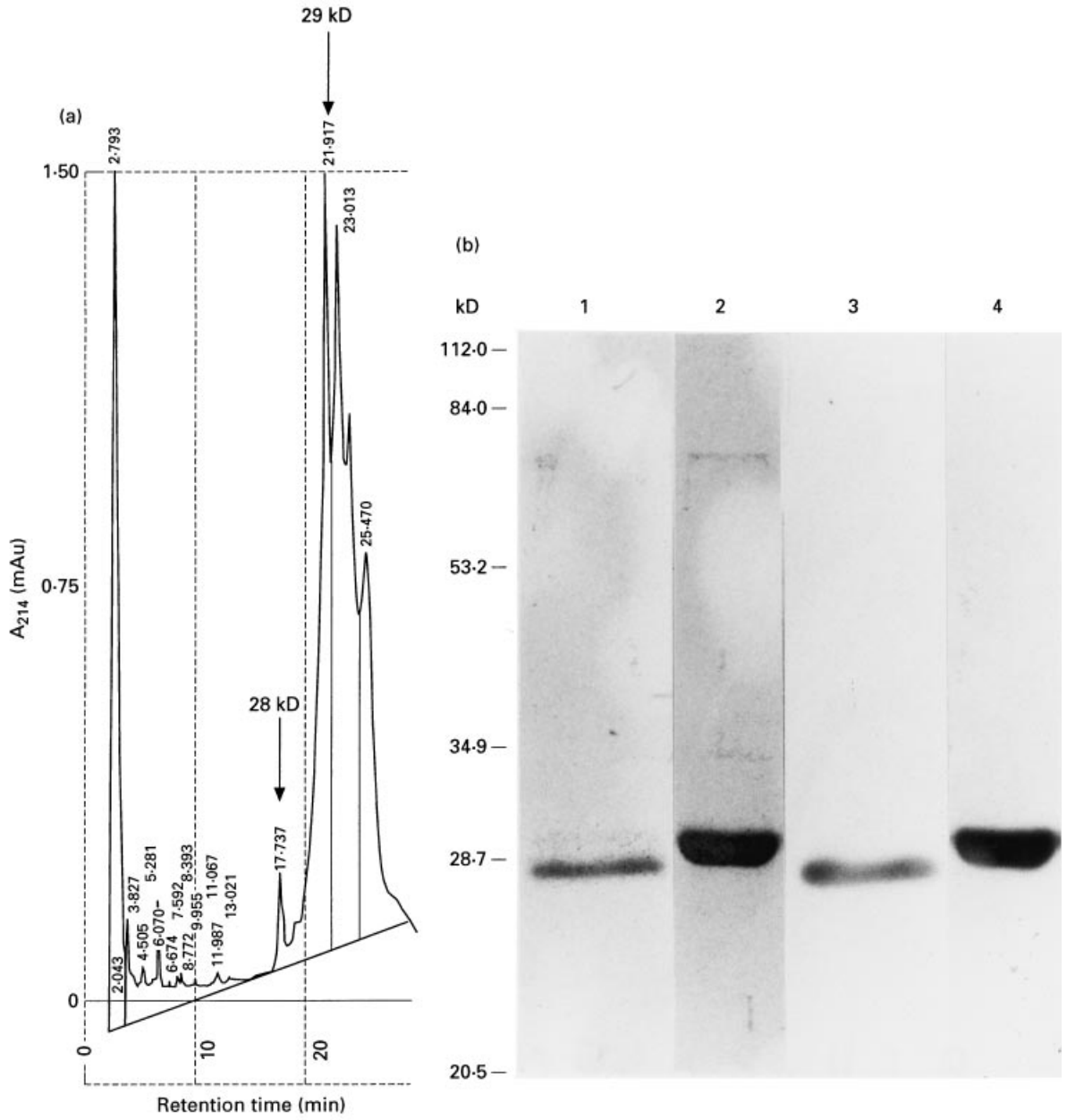

Fig. 2. Purification of the 28-kD antigen. (a) Reversed phase high-performance liquid chromatography (HPLC) profile. The peaks marked by arrows coincided with the 28-kD/29-kD antigens shown in (b). (b) SDS–PAGE stained by Fast stain (Zoion, Allston, MA) (lanes 1 and 2) and corresponding immunoblots (lanes 3 and 4) showing successive purification of the 28-kD/29-kD antigens.

analysis revealed that one protein was 28 kD and the other was 29 kD (Fig. 2b).

The portions of the membranes containing the antigens were cut off and subjected to amino acid sequence analysis without any modification of the thiol-residue of cystein. Direct sequencing of the intact protein was successful. The sequences of 32 N-terminal residues obtained are shown in Fig. 3. The only difference between the two proteins was residue 6, Asn for the 28-kD antigen and Lys for the 29-kD antigen. In both, residue 22 was not determined. Homology search, using the BLAST program of Altschul *et al.* [14] on the PIR database, revealed that the 28- and 29-kD proteins corresponded with residues 1–32, except residue 22, of HMG2 and HMG1, respectively. The striking compatibility in amino acid sequences and the same mobility on SDS–PAGE strongly suggested that the two proteins are HMG1 and HMG2.

Identification of the 28-kD protein

Alignment of the HMG1 and HMG2 family reveals a remarkable conservation in the primary sequence of the proteins [15–19]. There are differences in only two amino acid residues among human, porcine and bovine HMG1. The same number of differences are seen between human and porcine HMG2. Because of this high conservation in primary sequences, rabbit anti-porcine HMG1 and HMG2 antisera cross-reacted with human HMG1 and HMG2. This cross-reactivity enabled us to confirm that the HL-60-derived 28-kD/29-kD antigens are HMG1 and HMG2. These

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human HMG1      GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKT  50
29-kD           ....................X.........
human HMG2      .....N.........................S....A.............  50
28-kD           .....N..............X.........

human HMG1      MSAKEKGKFEDMAKADKARYEREMKTYIPPKGETKKKFKDPNAPKRPPSA  100
human HMG2      ......S.......S.....D....N.V....DK.G.K............  100

human HMG1      FFLFCSEYRPKIKGEHPGLSIGDVAKKLGEMWNNTAADDKQPYEKKAAKL  150
human HMG2      .......H.....S.........T.........SEQS.K......Q.....  150

human HMG1      KEKYEKDIAAYRAKGKPDAAKKGVVKAEKSKKKKEEEEDEEDEEDEEEEE  200
human HMG2      ................SE.G...PGRPTG....N.P.DE..E..E.D.D.  200

human HMG1      DEEDEDEEEDDDDE 214
human HMG2      E.......       208
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Fig. 3. Amino acid sequences of 28-kD/29-kD antigens. The sequences of the N-terminal 32 amino acids are illustrated, together with those of human high mobility group (HMG) 1 and HMG2, where dots indicate the homologous residues, and X, not determined.

antisera reacted not only with the HL-60-derived 28-kD/29-kD proteins but also with the 28-kD protein of neutrophil lysate (Fig. 4, lanes 7 and 5). Furthermore, among seven patients' sera which were positive for the neutrophil 28-kD band, six sera reacted with HMG1 and HMG2 purified from pig thymus [10]. One representative result is shown in Fig. 4 (lane 3). Normal human IgG, however, did not react with either porcine HMG1/2 or HL-60-derived 28-kD/29-kD antigens (Fig. 4, lanes 1 and 2). Moreover, the 28-kD band of neutrophil lysate that was probed by the patient's serum IgG disappeared after preincubation of the

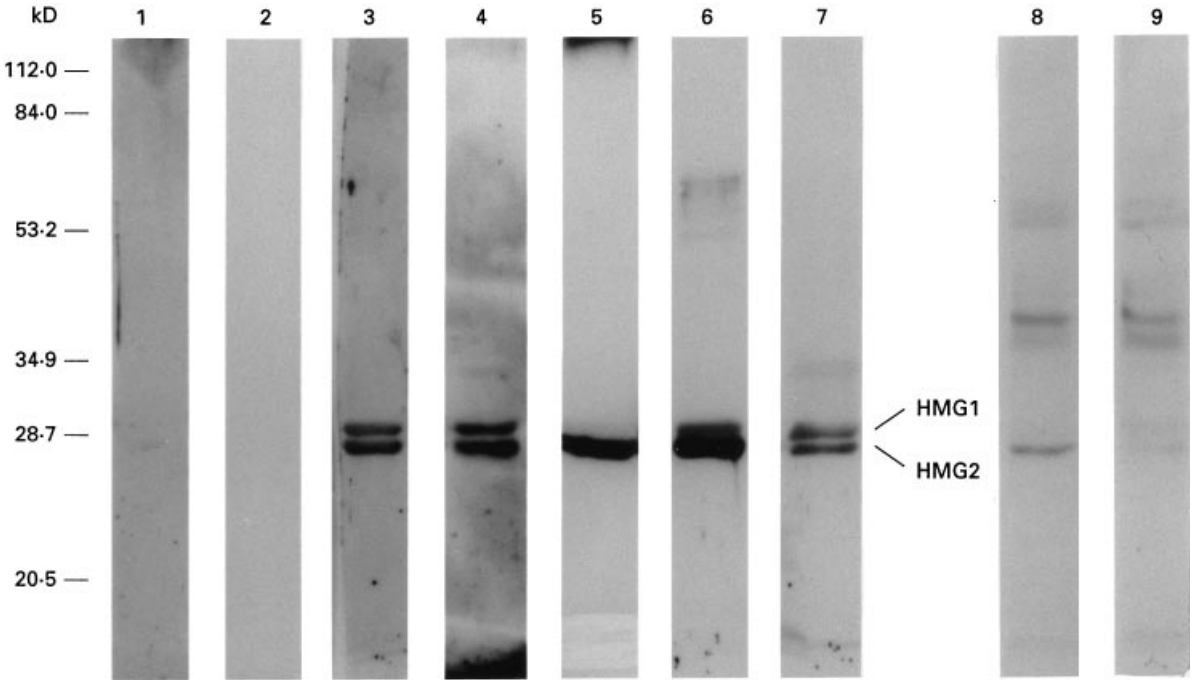

Fig. 4. Immunoblots demonstrating that the 28-kD neutrophil antigen recognized by the patient's IgG is identical to high mobility group (HMG) 1 and HMG2. Samples examined were porcine HMG1/HMG2 (lanes 1, 3 and 6), the 28-kD/29-kD antigens purified from HL-60 cells (lanes 2, 4 and 7) and neutrophil lysates (lanes 5, 8 and 9). The antibodies used were rabbit antiserum against porcine HMG2, which recognized both porcine and human HMG1/HMG2 (lanes 5–7), protein A-purified serum IgG from ulcerative colitis (UC) patients (lanes 3, 4 and 8) and that from healthy individuals (lanes 1 and 2). In lane 9, the patient's IgG (0·024 mg/ml) was preincubated with the mixture of bovine HMG1/HMG2 (1 mg/ml) at a ratio of 1:2 overnight at 4°C before immunoblotting.

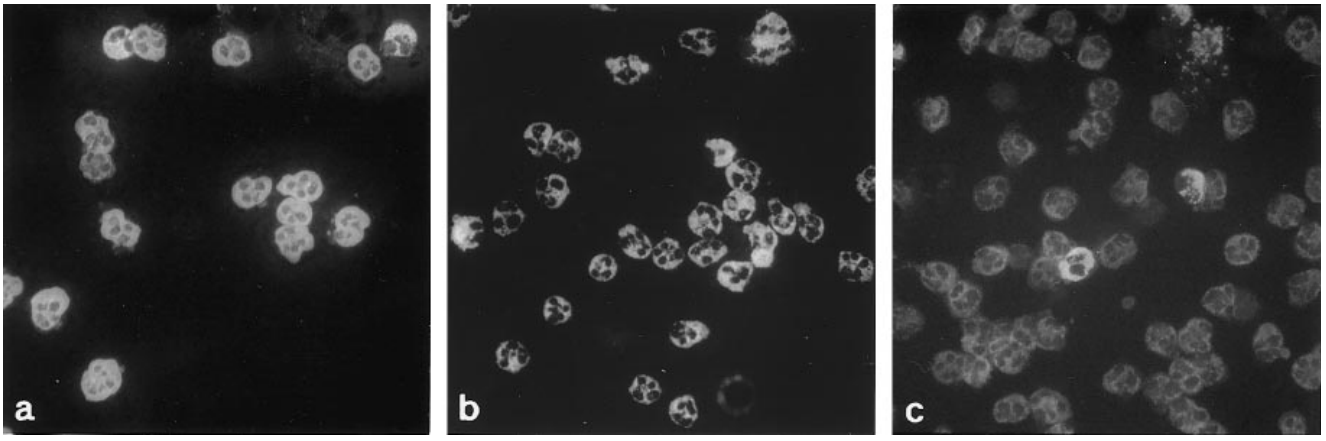

Fig. 5. Anti-high mobility group (HMG) 2 antiserum showed a P-ANCA staining pattern in indirect immunofluorescence. Samples were incubated with cytospin preparations of human neutrophils either ethanol-fixed (a and c) or formalin-fixed (b). The samples tested were rabbit antiserum against porcine HMG2 (a and b), and normal rabbit IgG (Dako, Glostrup, Denmark) (c).

patient's serum IgG with an excess of a mixture of bovine HMG1/2 (Fig. 4, lanes 8 and 9). This inhibition was dose-dependent (data not shown). These results clearly demonstrate that the neutrophil 28-kD protein is composed of HMG1 and HMG2.

IIF staining pattern

Anti-porcine HMG2 antiserum showed a perinuclear staining pattern for ethanol-fixed human neutrophils (Fig. 5a). Since the same antiserum showed a cytoplasmic staining pattern for formalin-fixed human neutrophils (Fig. 5b), the antiserum meets the criteria of P-ANCA [1,20]. Anti-porcine HMG1 antiserum showed similar staining patterns (data not shown).

DISCUSSION

In the previous study, we found that UC patients had a novel P-ANCA against neutrophil 28-kD protein. In this study, we purified the same antigens from HL-60 lysates by using reversed phase HPLC, and revealed that the 28-kD antigen consists of two different proteins. The N-terminus amino acids of these proteins are identical with those of HMG non-histone chromosomal proteins HMG1 and HMG2. Rabbit anti-HMG1/2 antiserum showed a perinuclear staining pattern in IIF studies using ethanol-fixed neutrophils. These data demonstrate that HMG1 and HMG2 are novel target antigens of P-ANCA in some patients with UC.

Several proteins have been identified as antigens of P-ANCA: myeloperoxidase, cathepsin G and lactoferrin [7,8,21]. All of these proteins are basic and located in the primary or secondary granules of neutrophils. In ethanol-fixed neutrophils, these proteins move to bind negatively charged nuclear membranes because of their positive charge, which results in perinuclear staining [1]. HMG1 and HMG2 are also positively charged (contents of Arg + Lys are 23·8% and 23·6%, respectively) except for the acidic C-terminus. These highly basic domains can allow them to move to bind the nuclear membranes in ethanol-fixed neutrophils. This may be the reason why the anti-HMG1/2 antisera exhibited a perinuclear staining pattern of ethanol-fixed neutrophils, whereas the same antiserum showed a cytoplasmic staining pattern of formalin-fixed neutrophils. These results clearly indicate that HMG1 and HMG2 can serve as P-ANCA antigens.

The presence of antibodies to HMG1 and HMG2 has been reported in sera of patients with systemic lupus erythematosus [22] and juvenile rheumatoid arthritis [23]. It is generally accepted that anti-HMG1/2 antibodies are antinuclear antibodies (ANA) [24]. However, these proteins are not exclusively distributed in the nucleus, but rather abundant in the cytoplasm [25,26]. In our study, anti-HMG1/2 antibodies did not stain the nucleus of ethanol- or formalin-fixed neutrophils on IIF. Previous studies also reported that anti-HMG1/2 antibodies stained predominantly the cytoplasm of formaldehyde-fixed HEp-2 cells [23] and methanol–acetone-fixed rat liver TR-12 cells [26]. Considering that anti-HMG1/2 antiserum showed a P-ANCA staining pattern of ethanol-fixed neutrophils on IIF, the pathological significance of anti-HMG1/2 antibodies should be studied in terms of not only ANA, but also ANCA. On the other hand, the intracellular localization and functions of HMG1 and HMG2 are distinct from those of the previously reported antigens for P-ANCA, which are peroxidase or proteolytic enzymes in neutrophil granules. These facts indicate that the criteria of ANCA should be discussed.

HMG1 protein stimulates transcription by modulating the structure of chromatin *in vitro* [27] and in cultured cells [28,29]. The amount of HMG2 protein parallels the proliferative activity of cells in various organs [30]. The level of HMG2 mRNA is enhanced in cells transformed with various viral genes [31], and cell growth is repressed by expression of antisense HMG2 RNA [31]. These findings indicate that HMG2 protein must be closely related to cell proliferation. On the other hand, the role of the antibodies against HMG1 and HMG2 is unclear. In unprimed neutrophils, HMG1 and HMG2 are present intracellularly, and therefore not accessible to circulating autoantibodies. Upon stimulation and rupture of the cells, these antigens may be exposed to P-ANCA, leading to an immune interaction. Immune complex formation might sustain or even amplify an inflammatory process. Alternatively, the released antigens may be internalized into the surrounding inflammatory cells and reach the chromatin to activate the genes, which may amplify an inflammatory process. Further investigations are necessary to study the putative pathogenic roles of such antigens and autoantibodies.

Although the significance of HMG1 and HMG2 in UC remains to be evaluated, it is noteworthy that the appearance of anti-HMG1/2 antibodies correlated with a refractory type of UC, as we reported previously ([9] and this paper). Considering that previously

identified P-ANCA in UC do not correlate with disease activity, duration, extent, or treatment [2,3,9], anti-HMG1/2 antibodies might become important in the serological diagnosis of refractory UC. It is also interesting that anti-HMG1/2 and anti-cathepsin G antibodies co-developed in patients with refractory UC [9]. To investigate the disease specificity of anti-HMG1/2 antibodies, screening of the antibodies by ELISA using HMG1 and HMG2 is underway.

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