Polymers of Z alpha-1 antitrypsin are secreted in cell models of disease

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Alpha-1 antitrypsin (A1AT) is a 52 kDa glycoprotein that is predominantly synthesised in the liver and secreted into the circulation, where it protects the lungs from the enzyme neutrophil elastase. Alpha-1 antitrypsin deficiency (A1ATD) is caused by mutations in the A1AT gene, with most cases resulting from homozygous inheritance of the Z allele (Glu342Lys). This leads to low levels of circulating A1AT, uncontrolled elastase activity and emphysema [1]. The Z mutation destabilises native A1AT and causes the formation of aberrant polymers that accumulate within the endoplasmic reticulum (ER) of hepatocytes, giving rise to inclusion bodies that are the main histological feature of A1ATD [2]. Extracellular polymers have been found in lung lavage, the skin of an individual with panniculitis and the kidney of an individual with vasculitis [1], and are also present in the circulation of all individuals homo- or heterozygous for the Z allele [3]. Circulating polymers originate in the liver, since they became undetectable in the plasma of an individual four days after liver transplantation [3], but it is unknown whether polymers can be secreted from hepatocytes or can form in the extracellular environment from secreted monomeric Z-A1AT. Extracellular polymers are chemotactic and stimulatory for human neutrophils [4] and may contribute to inflammatory neutrophil infiltration in the lungs, kidney and skin. It is important to understand where these polymers form in order to design effective therapies for emphysema and other pathological manifestations of A1ATD. Here we investigated the origin of extracellular polymers by exploiting our cellular models of A1ATD and conformer-specific and functional monoclonal antibodies (mAb) against Z-A1AT [5-8].

We first confirmed the presence of A1AT polymers in the plasma of three PiZZ individuals by non-denaturing PAGE and the polymer-specific mAb-2C1 [5] (Fig.1A). Similarly, when expressed in HEK293T cells Z-A1AT was detected in the culture medium as a mix of monomer and polymers (Fig.1B). To exclude the possibility that polymers were released into the culture medium from damaged cells, we analysed the glycosylation state of extracellular A1AT. Secretory glycoproteins are synthesised in the ER, modified in the Golgi apparatus where N-linked glycans increase in size and complexity, and arrive to the trans-Golgi network (TGN) for sorting and secretion. We

ascertained the state of N-linked glycosylation by treating M- and Z-A1AT with endoglycosydaseH (endoH, removes pre-Golgi glycans) or PNGaseF (removes all N-linked glycans) glycosydases. Digestion with endoH showed that all extracellular A1AT contained only mature, endoH-resistant N-glycans, which were removed by PNGaseF giving the expected size for non-glycosylated A1AT (Fig.1C), demonstrating that extracellular polymers bear mature N-glycans, resulting from their transport through the Golgi or from extracellular polymerisation of monomeric Z-A1AT, but not from direct release of immature ER proteins.

We next used our polymerisation-blocking mAb-4B12 [8] to prevent Z-A1AT polymerisation in cell culture medium. This antibody blocks polymer formation at 1:1 Z-A1AT:mAb-4B12molar ratio *in vitro*, so adding it in excess to the culture medium should inhibit polymerisation of secreted monomeric Z-A1AT. CHO cells expressing Z-A1AT under a Tet-inducible promoter [7] were cultured for 24h with or without mAb-4B12 at 1:1 or 1:2 molar ratio (Z-A1AT:mAb-4B12). There was no difference in polymeric Z-A1AT in the culture media by ELISA (Fig.1D), indicating that polymers were secreted from the cells. The presence of a complex between secreted Z-A1AT and mAb-4B12 was confirmed by ELISA, demonstrating binding of mAb-4B12 to secreted Z-A1AT (not shown). The secretory origin of extracellular polymers was further supported by the observation that culture medium from CHO cells expressing Z-A1AT induced for 48h and further incubated in the absence of cells for 72h showed no increase in polymer levels (not shown).

M-A1AT is readily secreted and can be detected by immunofluorescence within the Golgi compartment [5, 7, 9-11]. Although polymers of Z and other mutant variants of A1AT are found in the culture medium of expressing cells, they have never been shown to co-localise with Golgi-resident proteins [5, 7, 10, 11]. This may be due to very low levels of these proteins transiting the Golgi at steady state, and so we used a temperature block by culturing cells at 20°C, which reduces the exit of secretory proteins from the Golgi apparatus without affecting ER to Golgi transport [12]. Secretion of M- and Z-A1AT from transiently transfected COS-7 cells was reduced five- and two-fold upon incubation at 20°C for 4h (not shown). In these conditions, we assessed the co-

localisation of 2C1-positive polymers with resident proteins of the secretory pathway: BiP (ER), giantin (cis-medial-Golgi) and TGN-46 (TGN). The majority of 2C1-positive Z-A1AT co-localised with BiP, confirming polymer accumulation within the ER [5, 7], but we also observed partial co-localisation with giantin and TGN-46 (Fig.1E), supporting their transiting through the Golgi apparatus and exiting the cells *via* the canonical secretory pathway.

In order to confirm our results we used myc- and HA-tagged versions of M- and Z-A1AT (M_{myc}, Z_{myc}, M_{HA} and Z_{HA}) to transiently transfect HEK293T cells. We expressed each version of Z-A1AT or different mixes of the M- and Z-A1AT constructs, as described in figure 1F. We also created a new experimental condition by collecting, mixing and replating cells transfected separately with Z_{HA} and Z_{myc} (Fig.1F, G, lane 4). All the transfections were successful in producing similar amounts of A1AT (Fig. 1F, top panel), monomeric for the M-A1AT and polymeric for the Z-A1AT constructs (Fig.1F, bottom panel). We then performed immunoprecipitation of the culture media with anti-HA antibodies, followed by western blot with an anti-myc-tag antibody and an anti-totalA1AT antibody in the same membrane. All samples containing Z_{HA} gave a positive signal with the anti-A1AT antibody, demonstrating an efficient immunoprecipitation (Fig.1G, bottom panel), but only cells co-transfected with Z_{HA} and Z_{myc} showed a band positive to the anti-myc antibody, demonstrating the presence of Z_{HA}/Z_{myc} heteropolymers in their culture medium (Fig.1G, top panel). In contrast, cells transfected separately with Z_{HA} and Z_{myc} that were afterwards cultured together showed no signal with the anti-myc antibody. These results support the intracellular origin of secreted polymers.

Taken together, our results indicate that polymers formed within the ER can traffic through this organelle and be secreted *via* the Golgi compartment, where N-glycans acquire their mature configuration. Our biochemical and immunofluorescence results do not exclude polymer formation in post-ER compartments, but the existing literature and our present data are in keeping with polymerisation occurring mostly within the ER, where insoluble long-chain polymers are trapped within inclusion bodies, while at least part of the soluble, short-chain polymers can enter transport

vesicles for secretion. Our present observations suggest that polymers secreted from hepatocytes can contribute to circulating polymers. Although the concentration of monomeric Z-A1AT in plasma is probably high enough to allow extracellular polymerisation, we did not observe an increase in polymer levels when plasma samples from PiZZ patients were incubated at 37°C for 72h (Fig.1H). This can be due to limitations in our experimental conditions or to the presence of polymerisation-inhibitory factors in plasma. Our results are also compatible with local, extracellular polymer formation in the lung during inflammation and particularly by exposure to cigarette smoke [13]. In conclusion, our present results show for the first time that polymers of Z-A1AT are secreted from cells, supporting a contribution of intracellular origin to circulating polymers in A1ATD.

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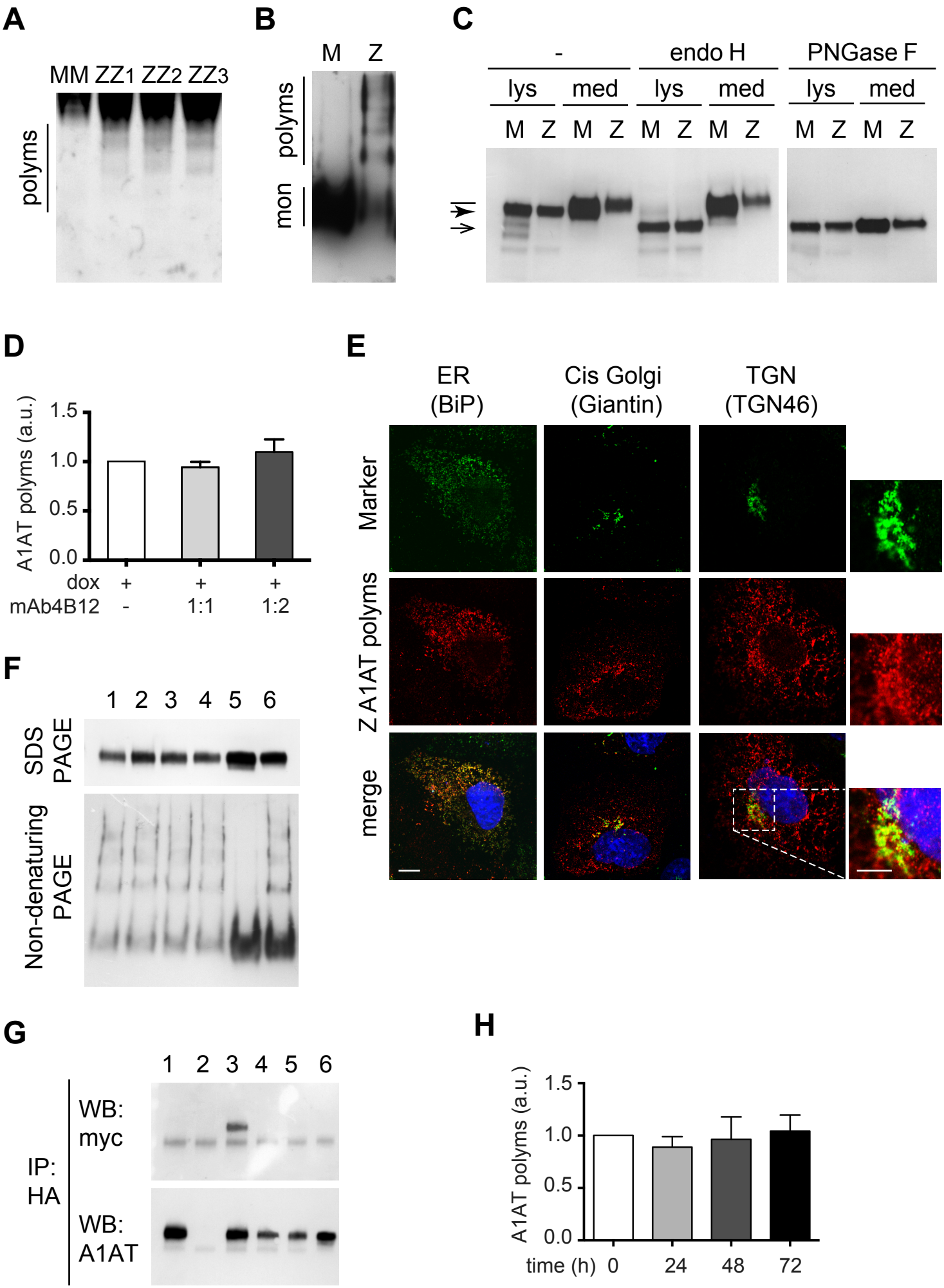
Figure 1. Extracellular polymers of Z-A1AT do not form in the extracellular medium, but are secreted from cells *via* the Golgi complex. (A) Polymers of Z-A1AT were found in the plasma of three PiZZ AATD individuals but not in plasma from a control donor by non-denaturing PAGE (stacking gel pH 6.8, separative gel pH 8.8) and western blot analysis using the polymer-specific mAb 2C1. (B) The culture medium of HEK293T cells expressing M-A1AT contained only monomeric protein (mon), while Z-A1AT was found as monomeric and polymeric (polyms) forms after non-denaturing PAGE and western blot analysis for total A1AT. The differences in band patterns and background levels between the polymer ladders in panels A and B are probably due to high protein concentrations in plasma samples compared to serum-free culture medium supernatants. (C) Cell lysates (lys) and culture media (med) of cells as in (B) were treated or not (-) with endoglycosidase H (endoH) or PNGaseF and analysed by SDS-PAGE and western blot for total A1AT. All extracellular A1AT carried mature N-glycosylation (55 kDa, line), while intracellular A1AT showed immature N-glycosylation (black arrow); the open arrow indicates deglycosylated A1AT. (D) Expression of Z-A1AT was induced in CHO stable cells with 0.5 μg/ml doxycycline (dox) and simultaneously incubated with purified mAb 4B12 at 1:1 or 1:2 (Z-A1AT to mAb 4B12) molar ratio for 24 h; the amount of extracellular polymers as quantified by sandwich ELISA (2C1-Ag-9C5-HRP) did not change in the presence of the polymerisation-blocking antibody. (E) Immunodetection of Z-A1AT polymers with mAb 2C1 (red) in co-localisation with resident proteins (green) of the ER (BiP), cis/medial Golgi (giantin) and TGN (TGN-46); the cell nucleus was counter-stained with DAPI; bars represent 10 and 5 μm in the main and close-up panels respectively. All panels were obtained in a Zeiss LSM510META confocal microscope with a 63x oil objective. (F) HEK293T cells were transiently transfected with vectors encoding Z_{HA} (lane 1) or Z_{myc} (lane 2), or co-transfected with Z_{HA}/Z_{myc} (lane 3), M_{HA}/M_{myc} (lane 5) or Z_{HA}/M_{myc} (lane 6); an additional condition was created by detaching, mixing and replating equal numbers of Z_{HA} and Z_{myc} cells (lane 4) at the end of the 5 h transfection. After an overnight incubation in normal culture medium, cells were further incubated for 24 h in serum-free medium,

and culture media were analysed by SDS- or non-denaturing PAGE. (G) Culture media as in (F) were immunoprecipitated with an anti-HA antibody and analysed by SDS-PAGE and immunoblot with an anti-myc-HRP mAb (top panel), then stripped and re-probed with the 2G7 anti-total A1AT mAb (bottom panel). (H) Plasma from four different ZZ patient donors was incubated for the times indicated at 37°C and analysed by sandwich ELISA with the 2C1 mAb. The graph shows the amount of Z AAT polymers normalised to time 0.

Figure 1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