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A copper–hydrogen peroxide redox system induces dityrosine cross-links and chemokine oligomerisation

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ABSTRACT

The activity of the chemoattractant cytokines, the chemokines, *in vivo* is enhanced by oligomerisation and aggregation on glycosaminoglycan (GAG), particularly heparan sulphate, side chains of proteoglycans. The chemokine RANTES (CCL5) is a T-lymphocyte and monocyte chemoattractant, which has a minimum tetrameric structure for *in vivo* activity and a propensity to form higher order oligomers. RANTES is unusual among the chemokines in having five tyrosine residues, an amino acid susceptible to oxidative cross-linking.

Using fluorescence emission spectroscopy, Western blot analysis and LCMS–MS, we show that a copper/H_2O_2 redox system induces the formation of covalent dityrosine cross-links and RANTES oligomerisation with the formation of tetramers, as well as higher order oligomers. Amongst the transition metals tested, namely copper, nickel, mercury, iron and zinc, copper appeared unique in this respect. At high (400 μM) concentrations of H_2O_2, RANTES monomers, dimers and oligomers are destroyed, but heparan sulphate protects the chemokine from oxidative damage, promoting dityrosine cross-links and multimer formation under oxidative conditions. Low levels of dityrosine cross-links were detected in copper/H_2O_2-treated IL-8 (CXCL8), which has one tyrosine residue, and none were detected in ENA-78 (CXCL5), which has none. Redox-treated RANTES was fully functional in Boyden chamber assays of T-cell migration and receptor usage on activated T-cells following RANTES oligomerisation was not altered.

Our results point to a protective, anti-oxidant, role for heparan sulphate and a previously unrecognised role for copper in chemokine oligomerisation that may offer an explanation for the known anti-inflammatory effect of copper-chelators such as penicillamine and tobramycin.

1. Introduction

The chemokines are a family of peptides that participate in the immune response at sites of inflammation, attracting sub-sets of leukocytes through activation of G protein-coupled receptors [1]. The heparan sulphate side chains of proteoglycans play multiple roles in the extravasation of leukocytes during the inflammatory response, and are abundantly expressed on endothelial cell surfaces [2]. Endothelial cell surface glycosaminoglycans (GAGs) bind chemokines, including RANTES (CCL5) and IL-8 (CXCL8), and induce their polymerisation [3]. The association of chemokines with heparan sulphate protects the peptides from proteolysis and stabilises tissue-bound concentration gradients, increasing the local concentration for presentation to leukocyte receptors [2]. Conversely, soluble GAG-chemokine complexes are biologically inactive [4]. Importantly, the activity of certain chemokines, including RANTES, *in vivo* is enhanced by oligomerisation and aggregation on endothelial cell surface GAGs [5].

Resonance of this scenario with the copper-induced formation of stable oligomers of Aβ [6], the principle component of β-amyloid deposits that are found in association with heparan sulphate in Alzheimers disease [7], led us to investigate the role of copper in oligomerisation of the chemokines. *In vitro*, stable oligomers of Aβ were formed by the copper/hydrogen peroxide-induced oxidative cross-linking of tyrosine residues [6]. Tyrosine residues are relatively rare in the primary sequence of chemokines, and we therefore focused our investigation on RANTES, a T-lymphocyte and monocyte chemoattractant, containing five tyrosine residues [8], which has a minimum tetrameric structure for *in vivo* activity [5] and a propensity to form higher order oligomers [9].

Copper is an essential trace element and cofactor for several enzymes that are important in normal cell function through its ability to change oxidation state from Cu^{2+} to Cu^{+} by acceptance of a single electron [10]. However, copper acquisition, distribution and use requires tight homeostatic regulation [10] as free copper in the human body, that which is not covalently bound to ceruloplasmin, poses a risk of Alzheimers and other neurodegenerative diseases,

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as well as inflammatory, fibrotic and autoimmune diseases, through its ability to generate reactive oxygen species (ROS) [10,11]. The release and activation of copper from ceruloplasmin is enhanced at acidic pH, which is a characteristic of sites of inflammation [12]. Copper has a number of pro-inflammatory effects, including activation of NF-κB [13] and expression of IL-8 and RANTES [14,15]. However, the role of copper in chemokine multimerisation has not previously been investigated.

In light of the above, we hypothesised that the redox activity of copper plays a role in the stable oligomerisation of certain chemokines via the formation of dityrosine cross-links, and that heparan sulphate promotes this activity.

We show that a copper/H_2O_2 redox system induces dityrosine formation and oligomerisation in recombinant human (rh) RANTES > IL-8 > ENA-78 (CXCL5), to an extent dictated by the number of tyrosine residues in the primary sequence, five in RANTES, one in IL-8 and none in ENA-78, and that heparan sulphate (HS) protects RANTES, and other chemokines, from oxidative damage.

2. Experimental procedures

2.1. Incubation of rh RANTES, IL-8 and ENA-78 with copper and hydrogen peroxide

Chemokines (Peprotech, London, UK) were incubated for 1 day (IL-8 and ENA-78) or 2 days (RANTES) at 37 °C in 0.5 ml Safe-Lock Eppendorf tubes at a final concentration of 5×10^{-7} M for the detection of RANTES and other chemokines, and at 2.5×10^{-6} M for the detection of dityrosines, in a final volume of 40 μl in the absence and presence of Cu, Ni, Hg, Fe or Zn chloride and H_2O_2 at the concentrations indicated. DMSO (1%, 5%, 10% v/v) was included in some incubations.

2.2. Western blot analysis of chemokine multimers

Chemokine multimers were separated on 14% SDS–PAGE gels, transferred to nitrocellulose membrane and the membranes blocked with 2% Tween-20. RANTES, IL-8 and ENA-78 were detected using biotinylated rabbit polyclonal anti-human RANTES, goat polyclonal anti-human IL-8 or rabbit polyclonal anti-human ENA-78 (Peprotech) at 0.1 μg/ml in PBS/2% (v/v) Tween-20, and then incubated with a 1:20,000 (v/v) dilution of a streptavidin–biotin horseradish peroxidase complex (StreptABC, DAKO, Ely, UK) for 45 min at room temperature, and blots developed using SuperSignal ECL kit (Thermo Scientific Pierce, Loughborough, UK) and placed against X-ray film to visualise results.

2.3. Analysis of dityrosines in RANTES multimers

A dityrosine-BSA conjugate was synthesised by cross-linking dityrosine (1.7 mg) with BSA (2.4 mg) in 0.85 ml 0.1 M phosphate buffer, with the addition of 200 μl 20% v/v glutaraldehyde, followed by dialysis against phosphate buffered saline (PBS). Dityrosines in the dityrosine-BSA standard and chemokines in solution were analysed by fluorescence spectroscopy at an excitation wavelength of 300 nm using a Varian fluorescence spectrophotometer and Cary Eclipse software. Analysis of dityrosines on Western blots used the peptidyl-dityrosine specific IC3 antibody as previously described [6,16]. Dityrosine standards were synthesised and LCMS-MS was carried out as previously described [17].

2.4. Isolation and activation of normal human T-cells

Whole blood was diluted 1:2 and layered on Lymphoprep (Axis-Shield, Dundee, UK), followed by centrifugation for 30 min at 438*g* at 20 °C. PBMC from the plasma/Lymphoprep interface were resuspended in RPMI/10%FCS supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B and depleted of monocytes by adherence overnight on plastic. Non-adherent T-cells were recovered and activated with PHA (1 μg/ml) for 2 days, followed by IL-2 (200 U/ml) for 3 days at 37 °C and 5% CO_2, (adapted from [18]).

2.5. Chemotaxis assays

Chemotaxis of activated T-cells in Hanks Balanced Salt Solution with Ca/Mg (Invitrogen, Paisley, UK) and 20 mM HEPES, pH 7.4, through 5 μm PVP-free uncoated polycarbonate filters was quantified using the modified micro-Boyden chamber technique (Neuro Probe, Gaithersburg, USA). Migrated cells adherent to the underside of the filter were stained with Hema-Gurr (VWR, Lutterworth, UK) and counted in five high power (×400) fields, (adapted from [18]).

RANTES samples and isolated T-cells were in incubated with 100 μg/ml anti-CCR3, anti-CCR5 neutralising antibodies, or rat IgG2a and mouse IgG2b isotype controls (R&D Systems, Oxford, UK) for 30 min before they were used in the chemotaxis assay.

2.6. Statistical analysis

Data were compared with a 1 or 2-way ANOVA followed by either a Dunnet's or Tukey's *post hoc* test where $p < 0.05$ was the minimum accepted level of significance. The Western blots are representative of at least two independent experiments.

3. Results

3.1. The copper/H_2O_2 redox system induces dityrosine formation and RANTES oligomerisation

In preliminary experiments, the chemokines were incubated at 5×10^{-7} M and at this concentration, in the absence of copper and hydrogen peroxide, SDS–PAGE analysis with Western blotting detected only 8 kDa monomers and 16 kDa dimers in every case (not shown).

The presence of copper (25 μM) alone had no effect on the oligomerisation of RANTES, (Fig. 1a, lane 1). However, in the presence of copper (25 μM) plus increasing concentrations 1–200 μM of H_2O_2 (Fig. 1a, lanes 2–8) an increase in the dimer/monomer ratio was observed, together with formation of higher order oligomers with molecular weights up to ca. 60 kDa, at the expense of the monomeric form. Most striking was the absence of detectable RANTES, monomer or multimers, in the presence of 400 μM H_2O_2 (Fig. 1a, lane 9). However, in the presence of heparan sulphate (HS), higher order oligomers were detected at 400 μM H_2O_2 (Fig. 1b, lane 9) with molecular weights up to ca. 100 kDa. In the absence of H_2O_2, HS alone had no effect on RANTES oligomerisation (Fig. 1b, lane1). Hydrogen peroxide alone at any concentration in the absence and presence of heparan sulphate had no effect. The multimers were stable, resistant to SDS in the gels, and also 2-mercaptoethanol and incubation with 6 M guanidine hydrochloride overnight, indicating formation of covalent linkages.

In separate experiments, we used scanning densitometry to analyse the quantitative changes in the oligomers present at concentrations of copper (25 μM) and hydrogen peroxide (50 μM), such as those detected at sites of inflammation. In the absence of heparan sulphate, 62.15 ± 9.7% of RANTES was present as oligomers greater than a dimer. In the presence of heparan sulphate (0.1 mg/ml) this was reduced significantly ($p < 0.1$, $n = 3$) to