Monocyte cytokine synthesis in response to extracellular cell stress proteins suggests these proteins exhibit network behaviour

Frank Kaiser · Andrew Steptoe · Stephen Thompson · Brian Henderson

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Abstract Human peripheral blood monocytes were exposed to single or pairs of cell stress proteins (CSPs), specifically Hsp10, Hsp27, Hsp60 and Hsp70—the former two having anti-inflammatory actions while the latter pair being assumed to be pro-inflammatory in activity. This study was to test if these proteins exhibited any network behaviour. To control for possible lipopolysaccharide contamination, polymyxin B was used. Surprisingly, at concentrations higher than 1 µg/ml, polymyxin B itself could induce cytokine synthesis. A number of commercial sources of the molecular chaperones were tested, and marked variations in monocyte cytokine synthesis were found. All four CSPs stimulated the same profile of IL-1/IL-6 synthesis and IL-10/TNF- α synthesis although the kinetics of production of these two pairs of cytokines were very different. A key question was whether extracellular molecular chaperones exhibited network behaviour. To test this, monocytes were cultured with suboptimal concentrations of single CSP and pairs of CSP to look for additive, synergistic or antagonistic cell responses. The major finding was that pairs of molecular chaperones, including chaperones thought to stimulate monocyte cytokine synthesis, could produce significant antagonistic cellular responses. This demonstrates that extracellular CSPs constitute an additional potent layer

within the complex cytokine network and furthermore suggests that monocytes have evolved to dampen their immune responses upon exposure to extracellular networks of CSPs—perhaps as a mechanism for protecting cells against detrimental cellular stress responses.

Keywords Cell stress proteins · Cytokines · Network behaviour · Inflammation

Introduction

Cell stress proteins (CSPs), a term that encompasses molecular chaperones and protein-folding catalysts, were initially thought to be intracellular proteins which functioned in the various cell compartments to control protein folding homeostasis (proteostasis) (Morimoto 2011). Their mode of action was to fold nascent proteins, refold unfolded proteins and solubilise protein aggregates in cells subject to stress (Hartl et al. 2011). At the time of writing of this paper, there are many distinct families of these proteins with, perhaps in humans, 100–200 separate CSPs (Calderwood 2007).

Contemporaneously with the discovery of CSPs as molecular chaperones (Hemmingsen et al. 1988) came the unexpected finding that these proteins could be secreted by cells (Tytell et al. 1986; Hightower and Guidon 1989) and that such secreted cell stress proteins were potent extracellular signalling molecules with macrophages (Sherry et al. 1992; Friedland et al. 1993) and lymphocytes (Tagaya et al. 1989). Indeed, 1 year before the introduction of the term ‘molecular chaperone’ in 1977, it was reported that women in the first trimester secreted an immunosuppressive factor into the blood. This was termed early pregnancy factor (EPF) (Morton et al. 1977), but it was not until 1994 that EPF was demonstrated to be the mitochondrial molecular chaperone, chaperonin 10 (Cavanagh and Morton 1994).

Since the discovery in the late 1980s/early 1990s that CSPs were secreted by cells and had intercellular signalling abilities,

F. Kaiser · B. Henderson
Department of Microbial Diseases, UCL Eastman Dental Institute,
London, UK

A. Steptoe
Epidemiology and Public Health, University College London,
London, UK

S. Thompson
Department of Rheumatology, King's College London,
London, UK

F. Kaiser (✉)
Eastman Dental Institute, University College London,
256 Gray's Inn Road, London WC1X 8LD, UK
e-mail: f.kaiser@ucl.ac.uk

it has been found that this is not just an isolated finding. At present, it is established that at least 16 CSPs are found in the human circulation (Henderson and Pockley 2012), and all of these proteins have some form of additional biological action (Henderson and Pockley 2010, 2012). Thus, these CSPs are examples of ‘moonlighting’ proteins, a term referring to proteins with more than one distinct biological activity (Jeffery 1999; Henderson and Martin 2011). Therefore, it would appear that in addition to their intracellular functions, largely concerned with protein folding, CSPs are secreted by various cell populations and have another set of functions including acting as intercellular signalling molecules. So far, the study of this signalling activity has concentrated on leukocytes, principally monocytes/macrophages. What is surprising is how much these CSPs appear to overlap with cellular signalling actions exerted by cytokines. Remarkably, the major measured product of cells upon stimulation with exogenous CSPs has been pro- and/or anti-inflammatory cytokines.

Cytokines are pleiotropic and pleiomorphic proteins with potencies in the nanomolar to femtomolar range (Henderson and Poole 1994). Simplistically, they can be categorised as pro-inflammatory or as anti-inflammatory, with TNF- α (Folmer et al. 2012) and IL-10 (Kubo and Motomura 2012) being the prototypic members for such immunoregulatory activities, respectively. Cytokines function largely as local paracrine and autocrine cellular regulators, and there is evidence that these proteins have complex behaviours with target cells and form what have been termed cytokine networks (e.g. Wilson et al. 1998). In the current context, a cell network can be thought of as a set of cells connected by one or more binary relationships which determine the influences (signals) between the cells. Signals may be multiple and include the property of aut signalling (autocrine modulation). Signals have an associated strength parameter which represents the relative importance of the signal to the cell. It needs to be appreciated that cytokine networks are dynamic entities in which connections and their strengths can change with time (Wilson et al. 1998). If network behaviour exists, it can produce a range of unexpected outcomes when cells are exposed to more than one stimulus. Examination of the potential network behaviour between IL-1 β , TNF- α and IL-10 reveals complex outputs when these three cytokines are modelled mathematically (Seymour and Henderson 2001). Notably, in *in vitro* studies of these cytokines, it was shown that unexpected relationships occurred in monocytes exposed both to IL-1 and TNF- α —in this case, synergistic behaviour (Stevens 2002)—or in animals exposed to both cytokines, where there is a synergistic increase in polymorphonuclear leukocyte accumulation (Henderson and Pettipher 1988). Antagonistic interactions can also occur (Wang et al. 2012).

Evidence is emerging that secreted CSPs have both pro- and/or anti-inflammatory actions. Thus, it is assumed that Hsp70 (HSPA1A) is a pro-inflammatory CSP (Asea et al.

2000), as is human Hsp60 (Kol et al. 2000). In contrast, Hsp10 (Johnson et al. 2005) and the small CSP, Hsp27 (De et al. 2000; Miller-Graziano et al. 2008), are both reported to have anti-inflammatory behaviour with human monocytes. However, it has recently been reported that Hsp27 stimulates the human monocyte cell line, THP-1, to upregulate NF- κ B and to enhance transcription of the genes encoding IL-1 β and TNF- α as well as IL-10 (Salari et al. 2013).

We have looked in more detail at the kinetics and dose responses of human monocytes exposed to these four molecular chaperones. It is possible that if cells secrete more than one cell stress protein, these proteins could exhibit network behaviour in the extracellular milieu. This possibility has been tested with a number of pairs of recombinant CSPs, and the evidence suggests that there can be marked interactions between different CSPs when they are used to modulate the activity of purified human peripheral blood monocytes.

Materials and methods

Reagents

Different preparations of recombinant CSPs (endotoxin—low grade for all protein preparations) were purchased from commercial suppliers: Hsp10 (Stressmarq SPR-310A, Stressgen SPP-110B, ATGen HSP0801), Hsp27 (Stressgen SPP-715D, ATGen HSP0503), Hsp60 (Stressmarq SPR-104A, ATGen HSP0802) and Hsp70 (Stressgen NSP-555D, ATGen HSP0603). Polymyxin B (tissue culture grade) was purchased as either sterile ready-to-use solutions (Sigma 92283, Calbiochem 420413) or as sulphate salt powder (Sigma P9423) dissolved in ultra-pure, endotoxin-free H₂O (PAA Laboratories) and sterile-filtered. Different polymyxin B preparations were initially compared and revealed similar behaviour (Fig. 1); accordingly, data shown in Figs. 2, 3, 4, 5 and 6 were generated using polymyxin B solution (92283) from Sigma. The lipopolysaccharide was from Cape Cod (*Escherichia coli* O113:H10) or Merck (*E. coli* O111: B4) and reconstituted in LAL reagent water (Cape Cod).

Isolation of human peripheral blood mononuclear cells

Peripheral blood from healthy donors was obtained by venepuncture. A written consent was obtained from each healthy subject, and all procedures followed were in accordance with institutional guidelines. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Lymphoprep (Axis-Shield, UK). After centrifugation, cells were washed twice in endotoxin-free sterile PBS (PAA Laboratories), and cell viability was routinely determined by trypan blue dye (Sigma) exclusion.

Fig. 1 High concentrations of polymyxin B induce cytokine secretion by human leukocytes in vitro. Freshly isolated human PBMC, 2×10^5 , were cultured in a 200- μ l medium (containing 10 % FCS) and pre-incubated for 15 min with different doses of polymyxin B (Sigma; 0.1–10 μ g/ml) before stimulation with different doses of LPS (Cape Cod; 1 pg/ml–10 ng/ml; shown as powers of 10). Cytokine production was measured in cell culture supernatants by ELISA after 24 h (mean $\pm$ SD; $n=3$). OD₆₀₀ readings were beyond the maximum detection levels of the ELISA assay and are therefore visualized on a log₁₀ scale axis (where OD₆₀₀=0.6 corresponds to 500 ng/ml of IL-1 or TNF- α , and OD₆₀₀=0.45 to 200 ng/ml of IL-6, respectively). A concentration of 1 μ g/ml of polymyxin B neutralised up to 10 ng/ml LPS while not stimulating itself the secretion of cytokines by PBMC

Purification of human monocytes

Monocytes were purified from freshly isolated PBMC by negative magnetic selection using the MACS Human Monocyte Isolation Kit II (Miltenyi Biotec). The purity of isolated monocytes (CD14⁺, CD3⁻, CD19⁻; all antibodies

from eBioscience, UK) was determined by flow cytometry analysis on a FACSCalibur (BD Biosciences) and was generally >95 % (data not shown).

Cell culture and in vitro cell stimulation

To facilitate adherence of human leukocytes to plastic surfaces of tissue culture plates, 2.0×10^5 cells/0.2 ml/well were seeded into 96-well flat-bottom plates (Sarstedt) in RPMI1640 (supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin; all from PAA Laboratories) in the absence of serum or plasma and incubated in a humidified 5 % CO₂ atmosphere at 37 °C. After 30 min, serum (FCS Gold, PAA Laboratories) or autologous plasma (for leukocytes or monocytes, respectively) was added to a final concentration of 10 % (v/v), and cells were rested overnight. The next day, the culture medium was replaced with fresh medium (containing 10 % (v/v) serum/plasma), and cells were stimulated with cell stress proteins in the presence of polymyxin B as indicated. Single doses of CSPs were normalised to their potency to stimulate similar TNF production by monocytes and, unless stated otherwise, used in cell culture at concentrations as follows: 5 μ g/ml Hsp10 (Stressmarq), 0.5 μ g/ml Hsp27 (Stressmarq), 0.1 μ g/ml Hsp60 (Stressmarq)

Fig. 2 While used at identical concentrations, different preparations of a recombinant cell stress protein can differ profoundly in their potency to induce cytokine production by human leukocytes. Freshly isolated human PBMC (1×10^6 cells/ml) were rested for 12 h and then stimulated in vitro with one dose (1 μ g/ml) of low-endotoxin preparations of recombinant CSPs (HSP10, HSP27, HSP60, HSP70) from different commercial suppliers (a, b, c) in presence of autologous plasma (10 %) and polymyxin B (Sigma; 1 μ g/ml). After 48 h of stimulation, production of IL-6 in cell culture supernatants was measured by ELISA. Similar data were obtained for other cytokines (IL-1, TNF- α , IL-10) (data not shown)

Fig. 3 Extracellular cell stress proteins induce cytokine secretion by human monocytes with distinct kinetic profiles. Highly purified human monocytes (1×10^6 cells/ml; purity >97 %) were rested for 12 h and then stimulated in vitro with one dose (1 μ g/ml) of recombinant CSPs in presence of autologous plasma (10 %) and polymyxin (1 μ g/ml). Cytokine production was measured from individual cell cultures for each time point by ELISA (mean $\pm$ SD; $n=3$). TNF- α levels at 24-h post stimulation were above the linear range of the assay, indicated with *dashed lines*

and 1 μ g/ml Hsp70 (ATGen), respectively. Cell cultures which have not been exposed to CSPs did not produce any measurable levels of cytokines and were included as negative controls throughout the study for each condition and time point tested (data not shown).

Analysis of cytokine secretion induced by cell stress proteins

Cell culture supernatants were harvested after indicated time points and stored at -20°C until analysis. Levels of IL-1 β , IL-6, IL-10 and TNF- α were measured by two-site ELISA using commercial kits according to the manufacturer's recommendations (Human Ready-SET-Go!® ELISA sets, eBioscience). Optical density of developed ELISA plates was measured using a microtitre plate reader (MRX II, Dynex) and cytokine concentrations calculated by plate

reader software (Revelation, Dynex). Each experimental condition was assayed by measuring triplicates of individual cell cultures, and experiments gave repeatable results.

Statistics

Analyses were performed using Prism software (GraphPad).

Results

High concentrations of polymyxin B induce monocyte activation

A commonly accepted standard control in studies investigating the effects of recombinant CSPs on eukaryotic cells is to

Fig. 4 Dose response of human monocytes to extracellular stress proteins. Highly purified human monocytes (1×10^6 cells/ml; purity >97 %) were rested for 12 h and then stimulated in vitro with different doses of recombinant CSPs in presence of autologous plasma (10 %) and polymyxin (1 μ g/ml). After 48 h of stimulation, the produced cytokines in cell culture supernatants were measured by ELISA. Dashed lines indicate the maximum detection level of the assay

add polymyxin B to the cell culture media in order to block any influence of bacterial lipopolysaccharide (LPS) which may contaminate recombinant proteins expressed in *E. coli* (Henderson et al. 2010). The literature commonly uses concentrations of polymyxin B up to 20 μ g/ml (Tabona et al. 1998). However, in the current studies, we found that concentrations of polymyxin B greater than 2 μ g/ml caused significant induction of pro-inflammatory cytokines in human leukocytes, most pronounced for IL-1 and IL-6, and to a lesser extent also TNF- α (Fig. 1). This was not due to a rogue batch of this protein as we tested several commercial polymyxin B products, and all generated the same profile of activity (data not shown). However, at lower concentrations between 0.5 and 2 μ g/ml, polymyxin B was still able to prevent LPS-induced cytokine secretion of PBMC, without itself causing cell activation (Fig. 1). Similar data were obtained when using purified human monocytes in this assay (data not shown). A low concentration of 1 μ g/ml polymyxin B was used in subsequent studies to neutralise any potential endotoxin contaminants in the preparations of recombinant CSPs.

Commercial recombinant cell stress proteins exhibit different potencies as extracellular signals

In the course of this study, recombinant CSPs were obtained from several different commercial suppliers (see “Materials and methods”). Testing these CSP preparations revealed that there were significant differences in the potency and efficacy of different batches of the same protein in their ability to induce cytokine synthesis by PBMC (Fig. 2).

Kinetics of monocyte cytokine synthesis in response to extracellular cell stress proteins

Highly purified human monocytes were exposed to CSPs (Hsp10, Hsp27, Hsp60, Hsp70) at a concentration (see “Materials and methods”) that induced a similar production of TNF- α by PBMC. Cells were then cultured in individual wells for each time point assessed, and the levels of cytokines were measured over 5 days. Unexpectedly, depending on the cytokine, each CSP produced a similar profile of cytokine synthesis 24 h post stimulation. However, cytokine synthesis and/or maintenance of cytokine levels over time differed significantly between the measured cytokines. Thus, TNF- α and IL-10 peaked at 24 h after CSP stimulation, and levels constantly declined thereafter. In sharp contrast, peak production of IL-1 and IL-6 was similarly detected at 24 h post stimulation, but then cytokine levels appeared to be stable until the last time point (d5) assayed (Fig. 3).

Dose dependency of monocyte cytokine secretion in response to cell stress proteins

If secreted CSPs exhibit some form of network behaviour, it will most simply manifest itself as either a synergistic or an antagonistic effect. In order to be able to determine these biological outputs, it is most convenient to set up experiments with individual CSPs which induce a response in a particular purified cell population, using CSP concentrations which are in the linear range of the dose–response graph and which produce less than 50 % of the maximal response. This is the most responsive part of the dose response allowing both potentiating or synergistic, as well as inhibitory or antagonistic, effects, to be elucidated. The dose–response

Fig. 5 The cytokine response of human monocytes exposed to cell stress proteins suggests network behaviour between individual cell stress proteins in the extracellular space. Highly purified human monocytes (1×10^6 cells/ml; purity >97 %) were rested for 12 h and then stimulated in vitro in presence of autologous plasma (10 %) and polymyxin (1 μ g/ml) with either one recombinant CSP (open bars) or a pair of two different CSPs (filled bars). Pro-inflammatory cytokine production in tissue culture supernatants was measured after 48 h of stimulation by ELISA (mean $\pm$ SD; $n=3$). Where indicated above filled bars, open brackets with dashed lines refer to the summation of the individual responses (open bars) to both CSPs

outputs of the various recombinant CSPs are shown in Fig. 4. This reveals that the dose-dependent responses of human monocytes differ: (1) depending on the CSP they are exposed to and (2) depending on the cytokine assayed. For most of the CSPs, the maximum concentrations tested apparently did not induce the maximum cell response in terms of cytokine production. Thus, the examination of pairs of CSPs at the concentrations chosen (see “Materials and methods”) should easily be able to encompass synergistic or antagonistic behaviours.

Fig. 6 The production of anti-inflammatory IL-10 by monocytes upon exposure to extracellular Hsp27 is inhibited in presence of other extracellular cell stress proteins. Highly purified human monocytes (1×10^6 cells/ml) were rested for 12 h and then stimulated in vitro in presence of autologous plasma (10 %) and polymyxin (1 μ g/ml) with either recombinant Hsp27 or other CSP family members alone (filled and open bars, respectively), or Hsp27 in presence of another CSPs (shaded bars). IL-10 production in tissue culture supernatants was measured after 48 h of stimulation by ELISA (mean $\pm$ SD; $n=3$). * $P < 0.05$; two-tailed Student's t test

Human monocytes exhibit a complex cytokine response to pairs of cell stress proteins

The addition of two different agonists to a cell will most likely result in an additive effect. Similarly, stimuli delivering cell inhibitory signals will most likely counteract the cell activating signals from agonists. When human peripheral blood monocytes were simultaneously exposed to two different CSPs, the presence of an additional member of the CSP family increased the production of pro-inflammatory cytokines as compared to the levels produced by the individual CSP (Fig. 5). Clearly, the ‘output’ (i.e. pro-inflammatory cytokine secretion) of two apparent distinct activating signals was not synergistic. Instead, while some combinations of distinct CSPs stimulated the secretion of pro-inflammatory cytokines in concentrations that correspond to additive levels of cytokines produced by individual CSPs, other pairs of CSPs elicited only up to half the amount of the projected calculated (i.e. additive) cytokine production, most pronounced for IL-1 (Fig. 5, Table 1). Interestingly, different pairs of CSPs demonstrated a varying degree of this effect, which, in addition, was specific for different pro-inflammatory cytokines.

The production of Hsp27-induced IL-10 by monocytes is inhibited in the presence of other cell stress proteins

In line with the described anti-inflammatory properties of Hsp27 (De et al. 2000), this protein induced the secretion of significant amounts of the key anti-inflammatory cytokine, IL-10, by cultured monocytes (Fig. 6). Intriguingly, upon costimulation with one other CSP, levels of IL-10 elicited by

Table 1 Additive behaviour of pairs of cell stress proteins

CSP pair	10+27	10+60	10+70	27+60	27+70	60+70
Cytokine						
IL-1	77	78	60	52	62	73
IL-6	111	106	82	91	100	125
TNF- α	93	91	115	99	99	68

Pro-inflammatory cytokine synthesis by purified human monocytes upon exposure to pairs of extracellular CSPs is expressed as a percentage of the summated cytokine synthesis stimulated by both CSPs individually

Hsp27 alone were reduced. This inhibition of an anti-inflammatory cytokine was in sharp contrast to the previously described behaviour of pairs of different CSPs for the induction of pro-inflammatory cytokines, where the presence of an additional CSP further increased cytokine levels produced by monocytes, albeit in many cases not in an additive fashion.

Discussion

It is now 23 years since the first report that thioredoxin had cell–cell signalling actions (Tagaya et al. 1989). This was only 1 year after Ellis and coworkers had established the basic rules for molecular chaperones based on the identification of the prototypic protein-folding protein, chaperonin 60 (Hemmingsen et al. 1988). In the 2010s, a significant number of cell stress proteins have been shown to function as extracellular signals with various leukocyte populations and subpopulations and with other cell populations such as: vascular endothelial cells, smooth muscle cells, fibroblasts, various bone cell populations and epithelial cells (Henderson and Pockley 2010, 2012). The significance of the cell–cell signalling properties of extracellular cell stress proteins only becomes important when it can be established that these proteins are secreted in the living organism and can exist in the extracellular milieu. Since the late 1990s, it has been shown by an increasing number of investigators that a growing number of the best known cell stress proteins, including chaperonin 10 (HSPE1), Hsp27 (HSPB1), chaperonin 60 (HSPD1), thioredoxin, peroxiredoxin, Hsp70 (HSPA1A), BiP (HSPA5) and Hsp90 (HSP90AA1), are present in the human circulation (Pockley and Frostegård 2005; Pockley and Multhoff 2008; Henderson and Pockley 2012). There is mounting evidence that levels of certain of these proteins in the circulation can either promote (e.g. Hsp60; Xu et al. 1999) or protect (e.g. Hsp27 Rayner et al. 2010) against cardiovascular disease. Moreover, there are now a number of human cell stress proteins/peptides in clinical trial such as Hsp60 peptide p277 for early onset diabetes (Huurman et al. 2008) and chaperonin 10 for a number of inflammatory conditions (Williams et al. 2008). These clinical trials show

that cell stress proteins can have major effects on cellular behaviour in a physiopathological context and that these proteins, once secreted, can have prominent biological effects on both health and disease.

Now, it is likely that, like cytokines, cells will secrete more than one cell stress protein and that these proteins could interact with the producing cell population. The authors have recently found that in participants of the well characterised Whitehall II cohort, a longitudinal study of cardiovascular risk factors in healthy UK civil servants (Stringhini et al. 2012), levels of three cell stress proteins, Hsp27, Hsp60 and BiP, can be measured in the circulation. Markedly, there was a strong positive correlation between the levels of these proteins in the participants, suggesting some form of network behaviour (unpublished data). For these reasons, we decided to determine if there was any evidence of network interaction between pairs of cell stress proteins, when they interact with human monocytes, and we picked a small number of relatively well-studied proteins: Hsp10, Hsp27, Hsp60 and Hsp70 for this purpose. From the literature, both Hsp10 (Johnson et al. 2005) and Hsp27 (De et al. 2000) have an anti-inflammatory profile of activity with human monocytes. In contrast, Hsp60 and Hsp70 are assumed, from the literature, to be pro-inflammatory proteins (Henderson and Pockley 2010).

All studies employing recombinant proteins, no matter the host strain, should control for possible contamination with the Gram-negative bacterial outer cell membrane component, LPS. One of the important controls is the addition of the LPS-binding and neutralising antibiotic, polymyxin B (Henderson et al. 2010). It therefore came as a surprise when we discovered that the polymyxin B we were using was actually stimulating human leukocytes that we were using in these studies to produce cytokines. This was not due to contamination of the polymyxin B preparation that we were using as we found the same effects with a range of commercial polymyxin B preparations. Dropping the polymyxin B concentration from 20 $\mu\text{g/ml}$ (which is often used in the literature) to 0.5–1.0 $\mu\text{g/ml}$ solved this problem in that the polymyxin B was still capable of inhibiting LPS-induced cell activation but without the capacity to stimulate cytokine synthesis in its own right. Perusal of the early literature

reveals a number of reports that ‘high’ concentrations of polymyxin B activate macrophage cytokine synthesis (Cavaillon and Haeffner-Cavaillon 1986; Damais et al. 1987; Høgåsen and Abrahamsen 1995), so these results are not novel but again emphasise the importance of checking each component in the cell-based assays that are being used to assess CSP agonist actions.

To determine how CSPs interacted with human peripheral blood monocytes singly and in pairs, it was important to first establish the biological potencies of these proteins. To this end, we purchased commercial high-grade preparations of recombinant proteins from various suppliers. To our surprise, there were marked differences in the biological potencies of these commercial recombinant proteins. This seemed most marked with Hsp27 preparations, which ranged from samples that had virtually no ability to induce human primary monocyte cytokine synthesis to those that had significant agonist activity with these cells. This was not due to contamination with biological PAMPs such as LPS as assessed by the use of the LPS-binding/neutralising antibiotic, polymyxin B. It is not clear what is responsible for the major differences in the biological activity of these CSP preparations. The simplest explanation would be that the inactive preparations contained mainly denatured protein. Alternative explanations may relate to the physicochemical state of the recombinant protein. For example, Hsp27 can exist in various oligomeric forms and in various phosphorylated states (Arrigo 2011). This difference in the agonist activities of recombinant Hsp27, and in other commercial CSP products, is of concern and needs to be taken into account when one is embarking on studies of the extracellular signalling actions of these proteins.

To understand the potential network behaviour of CSPs, it was important to establish the kinetics of monocyte cytokine synthesis induced by CSPs and also the biological potencies of these proteins. To our knowledge, there have been no comparative studies of cell stress proteins as monocyte cytokine inducers. When we examined the kinetics of cytokine synthesis in response to the four CSPs, it appeared that they all generated a similar kinetic but that this depended on the cytokines being assayed. TNF- α and IL-10 synthesis rapidly increased upon stimulation with CSPs, followed by a constant decline in cytokine levels over the next 5 days of cell culture. In contrast, the levels of IL-1 β and IL-6 did not decline precipitately after the peak at 24 h. It is not clear what is responsible for the loss of the immunoreactive signal of TNF- α and IL-10. CSPs may differentially modulate the internalisation or degradation of various cytokines by stimulated cells. What is interesting is the similarity in monocyte response to these very different proteins. A similar finding was noted when we examined the dose response of these four cell stress proteins. The dose dependency of the synthesis of each cytokine showed a different pattern, but for each

cytokine, all four cell stress proteins induced a similar dose–response relationship.

The key purpose of this study was to determine the response of human circulating monocytes to individual, versus pairs, of CSPs, in order to reveal first insights into how the complex composition of various CSPs found in the circulation may compose a network to control leukocyte function. This is the simplest system for understanding network behaviour, as the complexity of networks increases dramatically as the number of network elements (e.g. cell stress proteins) increases (Emmert-Streib and Dehmer 2012). It was expected that pairing two ‘pro-inflammatory’ cell stress proteins should give rise to, at the very least, additive behaviour and, if network behaviour was being induced, should result in a synergistic effect on the monocytes. Equally, the pairing of two anti-inflammatory CSPs should produce a negative synergy (antagonism). The pairing of an anti-inflammatory and a pro-inflammatory CSP should generate some form of additive behaviour, ultimately nullifying the cellular response.

To study the biological effects that pairs of CSPs, in comparison to single CSPs, have on leukocytes, we used concentrations of these proteins that generate less than 50 % of the maximum response recorded in the dose–response experiments. This was to allow for the determination of putative synergistic or antagonistic effects. With the various combinations of CSPs used, we expected to see at least additivity of response. However, this was only seen with certain CSP pairings. Instead, a marked number of pairings resulted in the synthesis of cytokines being significantly less than the summation of the response of monocytes when exposed to the respective CSPs individually. The magnitude of this effect seemed to differ for different cytokines and was most pronounced for IL-1 synthesis. This output was particularly marked with Hsp27 and IL-10 synthesis, where the net amount of IL-10 stimulated by Hsp27 alone was inhibited in the presence of an additional CSP protein family member.

These data suggest that monocytes respond to single cell stress proteins in a fairly uniform way which is surprising as the four proteins chosen in this study have been previously reported to have distinct effects on monocytes (Henderson and Pockley 2010). Does this mean that monocytes perceive all CSPs as generating the same ‘signal’ irrespective of their molecular nature? Hence, our future studies are aiming to gain further insights into the molecular mechanisms which determine the yet to be defined complex biological activity profile of CSPs in the extracellular space. Most instructive information may be obtained by examining which intracellular signalling pathways downstream of putative HSP receptors are being activated by different extracellular CSP family members on their own or in combination. Preliminary data suggest that exogenous CSPs stimulate a number of well characterised cell signalling pathways

involved in cytokine gene transcription and/or (post-)translational control, and it is tempting to speculate that individual CSPs share or differentially use such signalling cascades, which may form the molecular basis for a complex extracellular CSP network for leukocyte function control.

When exposed to pairs of CSPs, the most obvious expected result would have been a cytokine output that was the summation of both individual outputs for the single stimuli. However, for a number of pairings, the output of the pairs was significantly lower than the expected summation of the stimuli. There was no evidence for any synergistic interactions between the tested CSPs. This suggests that monocytes have evolved to downregulate their activation status when faced with more than one CSP in their external milieu. This is a reasonable strategy and would inhibit the consequences of monocyte over-activation when cell, organ or organismal stress is occurring and leading to the release of these potent signalling molecules.

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