Reviews

Functional and clinical aspects of the myelomonocyte protein calprotectin

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Calprotectin is a calcium binding heterocomplex protein consisting of two heavy and one light chain.[1 2] It belongs to the S-100 protein family and is derived predominantly from neutrophils and monocytes.[1] This protein is distributed in myelomonocytic cells, epithelial cells, and keratinocytes and in various tissues and fluids in the body,[1] and is a putative protective protein.[3 4] Calprotectin and its subunits appear to have regulatory functions in the inflammatory process,[5 6] and various biological functions including antimicrobial[7–9] and antiproliferative[10 11] activity have been ascribed to the protein. In acute phase inflammatory reactions calprotectin is detectable in elevated amounts that, in some instances correlate to elevated levels of neutrophil granulocytes or other inflammation parameters such as C reactive protein (CRP) or erythrocyte sedimentation rate (ESR).[12–14] Sander *et al*[15] found poor correlation between calprotectin and CRP, blood leucocytes, and ESR in life threatening infections, thus suggesting that these parameters reflect separate aspects of the inflammatory response.

The clinical relevance of calprotectin measurements has been described in several disease conditions, particularly in inflammatory diseases and certain microbial infections, as well as neoplastic conditions.[16 17] The highest rise in calprotectin concentrations can be found in cystic fibrosis, rheumatoid arthritis, Crohn's disease, ulcerative colitis, and bacterial infections.[12–14 18 19]

Why do we find calprotectin so fascinating? There are already numerous markers of inflammatory reactions[9 20–26] including granulocyte markers, so why introduce another? Calprotectin is a multipotent biologically active molecule.[1 5] We know that large molecules can have separate domains with different biological functions[27 28] and that cellular functions (in macrophages) can be selectively and simultaneously up or downregulated via different entities on such macromolecules.[28] Calcium binding proteins of the S-100 protein family are involved in complex intracellular signal tranductions,[27 29] and it has been suggested that calprotectin plays an important role in the metabolism of myeloid cells.[30] Furthermore, when calprotectin is external to cells it has immunomodulatory functions[5 22] and an important role in neutrophil defence against microbial infections.[21 31 32] Antitumour activity has also been suggested,[10 11] as well as an innate defence function.[3 4] Thus, calprotectin appears to be an important regulatory protein inside the myeloid cells and extracellularly in inflammatory reactions. This remarkable spectrum of functions demonstrated in a recently discovered protein prompted an up to date review as a basis for further studies.

Table 1 Calprotectin nomenclature

Nomenclature	*References**
Calprotectin (L1) (2 heavy + 1 light chain)	Fagerhol,[1 2] Fagerhol *et al*[33]
MRP 8/14 (MIF related protein)	Odink *et al*[34] Sorg[5]
Cystic fibrosis associated antigen (CFA) (identity with calprotectin and MRP-8)	Wilson,[35 36] Dorin[37] (Anderson *et al*[38 39])
Calgranulins A and B	Wilkinson *et al*[40]
S-100a and b (calcium binding proteins)	Dorin *et al*,[37 41] Freemont *et al*[27]

*First publications and/or key references.

Nomenclature

Calprotectin is an elusive protein with many names[2]; several independent research groups have called it L1 protein, MRP-8/14, calgranulin, and cystic fibrosis antigen (table 1).

Calprotectin was first isolated from granulocytes as described by Fagerhol *et al* in 1980[33] and named L1 protein. The name calprotectin was proposed later[8] when calcium binding and antimicrobial activity had been documented. Sorg's group[5] worked with macrophages and mechanisms of chronic inflammation, and the MIF related proteins MRP-8, MRP-14, and their heterocomplex MRP-8/14 were described in 1987 by Odink.[34] A cystic fibrosis associated antigen was first described by Wilson *et al* in 1973[35] and further characterised by Wilkinson *et al*[40] who proposed the name calgranulin in 1988. The identity between the L1 protein, cystic fibrosis antigen, and MRP-8 and MRP-14 was established in 1988 by amino acid and cDNA sequencing, and immunohistochemical staining.[38 39] Dorin *et al*[37 41] and Freemont *et al*[27] further characterised the S-100 structure of these proteins.

Calprotectin is a suitable descriptive name for this multipotent calcium binding protein with protective properties.[2]

Table 2 Physicochemical properties

Molecular weight 36 kDa[2]
Two heavy chains of 14 kDa[2]
One light chain of 8 kDa[2]
Each chain binds two calcium ions[42]
Zinc binding[43]
Heat resistant[42]
Resistant to proteolysis when calcium is present[1 2]
Immunogenic[44]
MoAb defined epitopes
Mac387[45-47]
27E10[5]
S 36.48[48]
S 32.2, 8-5C2[49]
CF145, CF557[46]
F11, F3, A1[50]
CP-1, -2, -5[51]
Strong bond between subunits[1 2]
Forms noncovalent complexes (-di, -tri, and tetramers) in a calcium dependent manner[2 3 52]
S-100 protein structure and sequence identity[1 17 37 53-55]
Mapped to human chromosome 1 (and murine 3)[41 48 56]

The references cited are representative reports or reviews, not a complete list of publications on each topic.
MoAb, monoclonal antibody.

Table 3 Distribution in cells and tissues

Cells
Neutrophil granulocytes[33 57 58]
Monocytes/macrophages[9 34 54]
Epithelial cells[20 60-62]
Keratinocytes[63-65]
Pancreatic cell lines[66]
Tracheal gland cells[67]
Tissues
Skin (epidermis/dermis)[62 68]
Lung[16]
Gut[69]
Oral mucosa[63 64]
Cervix mucosa[65]
Body fluids[1] (see table 6)
Other species
Mouse[70]
Rat[10 71]
Pig[72]
Sheep[73]
Rabbit[74]

The references cited are representative reports or reviews, not a complete list of publications on each topic.

Structure and physicochemical properties

The physicochemical properties of calprotectin (table 2) have been reviewed thoroughly.[1] It is a 36 kDa heterotrimeric calcium binding protein with two heavy and one light chain noncovalently linked.[1 2 42] In the presence of EDTA it is anionic and migrates in electrophoresis as an α_2 globulin, whereas in the presence of calcium it is slightly basic and migrates as a γ globulin. Free heavy and light chains have been detected only after dissociating treatment, typically by heating in the presence of sodium dodecyl sulphate (SDS) and 8 M urea followed by SDS polyacrylamide gel electrophoresis or two dimensional electrophoresis (isodalt). The polypeptide chains may be purified by isoelectric focusing in the presence of 6 M urea.[57 58] Reactive sulphydryl groups are exposed after dissociation, and unless these are blocked or alkylated, various heterodimers may be formed.[52 59] Addition of calcium may promote formation of such complexes and even calprotectin dimers—that is, double heterotrimers.[57 42]

Epitope mapping of the protein, by use of a series of overlapping seven amino acid long synthetic peptides covering the heavy and light chains, has shown that rabbits produce antibodies against three different linear epitopes on each chain (Hansen *et al*, 1995, unpublished). Many monoclonal antibodies against calprotectin have been produced (table 2). Epitope mapping of calprotectin with six murine monoclonal antibodies revealed four separate epitopes (Johne and Hansen, 1995, unpublished). Binding of calcium causes conformational changes in calprotectin as demonstrated by circular dichroism[42] and reactivity with monoclonal antibodies.[51] Furthermore, calcium makes the protein remarkably resistant against heat and proteolysis.[2 42] Its S-100 like protein structure has been established[1] and the genes have been sequenced and mapped to human chromosome 1, q12–q21.[41 48]

Distribution in cells and tissues

Calprotectin is found in cells, tissues, and fluids in all parts of the human body (table 3). It is mainly a myelomonocyte and keratinocyte protein. In neutrophils, calprotectin is located in the extralysosomal cytosol in concentrations estimated at 5–15 mg/ml,[33 57] thus constituting about 5% of total proteins in neutrophil granulocytes. By immunoelectron microscopy, small amounts were also found in electron dense parts of myelomonocyte nuclei (Stäubli and Fagerhol, 1991, unpublished). The calprotectin chains are expressed in monocytes and activated macrophages but in decreasing amounts with increasing differentiation of the latter. Calprotectin is variably expressed on the surface membrane of granulocytes and monocytes.[75] Dendritic cells may be induced to L1 expression in certain reactive states.[1] Various mucosal squamous epithelia have been shown to express cytoplasmic calprotectin even in the normal state.[60] Merten and Figarella[67] reported calprotectin secretion in tracheal gland cells, and Fanjul *et al*[66] demonstrated MRP-8 and MRP-14 in pancreatic cells. Calprotectin is also found in rat, mouse, rabbit, sheep, cattle, and pig.[10 70-74 76] It is thus an abundant ubiquitous molecule.

Biological function

Recent publications have revealed numerous biological functions of the calprotectin molecule in vitro and in vivo (table 4). The structural identification as an S-100-like protein and its calcium dependent association to cytoskeleton structures[29] suggests intracellular signal transduction functions.[87] Translocation has been shown of phosphorylated calprotectin chains to the membrane during human neutrophil activation,[88] and inhibition of intracellular enzymes important in cell proliferation (casein kinase II and topoisomerases).[89] It was demonstrated that the protein is phosphorylated, and this raises the possibility that calprotectin may be a competing substrate for the enzyme. The first documentation of antimicrobial activity[8] has been confirmed in several papers (table 4), and has given calprotectin a central role in neutrophil defence.[21 90 91] The abundance and distinctive properties of calprotectin suggests that it plays an important role in neutrophil biology.[32 92] Stimulation of immunoglobulin production,[6] chemotactic factor activity,[83] and

Table 4 Biological functions

Intracellular signal transduction (S-100 function)[27 47 77]
Calcium dependent association to cytoskeleton structures[29 77]
Antimicrobial activity[8 21 31 78 79]
Anti *Candida albicans* activity[33 80-82]
Neutrophil defence mechanism[21 32]
Stimulation of immunoglobulin production[5]
Chemotactic factor[83]
Neutrophil immobilising factor[27]
Regulatory protein in inflammatory reactions[5]
Marker of myelomonocytic cell differentiation[84 85]
Cytotoxic effects:
a) cytotoxic factor in rat peritoneal excudate cells[10]
b) induction of apoptosis[11]
Protective action against rheumatoid arthritis (rat model)[86]

The references cited are representative reports or reviews, not a complete list of publications on each topic.

neutrophil immobilising factor activity[27] are also functions in a non-specific defence repertoire, and related to its properties as a regulatory protein in inflammatory reactions.[13] Calprotectin is a marker for myelomonocytic cell differentiation,[84 85] and it has cytokine-like effects.[10 11] Brun *et al*[86] demonstrated protective action of injected calprotectin in a rat model of avridin induced rheumatoid arthritis, confirming its regulatory role in inflammatory reactions. Sorg[5] discussed the importance of the single protein chains versus the heterocomplex in chronic inflammation.

Data by Hahn and Sohnle,[9] Clohessy and Golden,[81 82] and Sohnle *et al*[93] suggest that the antimicrobial action is at least in part the result of binding to zinc. It has been shown[89] that calprotectin may inhibit metalloproteinases, which may also involve deprivation of zinc. The zinc binding site is separate from the calcium binding sites on MRP-14.[43] Thus calprotectin is a large multipotent biologically active molecule with defined regions or epitopes with distinct functions. There is, however, much to be done regarding the investigation of structure effect relations. Particularly exciting are the conformational changes related to calcium binding, and its importance for molecular functions.

Clinical relevance

The mere fact that no calprotectin deficiency has been found (among more than 5000 individuals tested) may suggest that this protein is of vital importance. The clinical relevance of calprotectin in a number of disease conditions has been suggested by several authors (table 5). The first measurements of concentration[33] in plasma from normal subjects and from patients with inflammatory, infectious, and malignant diseases have been followed by extensive documentation with immunohistochemical methods, and with immunometric concentration measurements in various body fluids (table 6). In several disorders the differences between normal and pathological levels are large enough to suggest a diagnostic potential for calprotectin.[15 17 18 97 105 106 118]

Early attention was paid to rheumatic diseases.[13 35 94] Cystic fibrosis was studied before the protein was identified and characterised, and several other inflammatory diseases have since been studied (table 5). The relevance of calprotectin in malignant diseases has also been pointed out.[16 17 50 123]

CALPROTECTIN IN PLASMA

Increased concentration of calprotectin in plasma is found in many types of infectious or organic diseases.[15] This seems logical in view of the large amounts of the protein in myelomonocytic cells, their active role in defence against infections, and their participation in inflammatory processes and removal of dead cells. We therefore hypothesised that plasma calprotectin concentrations reflect the turnover of myelomonocytic cells in the body. Plasma concentrations during viral infections rarely exceed 2 mg/l, while in bacterial infections values above 3 mg/l are seen regularly.

In a series of patients with meningococcal infections, calprotectin concentrations at hospital admission were between 10 and 120 mg/l in those with fulminant septicaemia (table 7). As calprotectin is preformed and ready to be released from activated circulating leucocytes, its concentration in plasma can increase much more rapidly in response to bacteraemia and endotoxaemia than acute phase proteins (CRP) synthesised in the liver. The latter has a doubling time of three to four hours, so it may take from six hours to more than a day for the plasma concentration to reach more than the upper reference limit. If a rapid test is developed for use in primary health care, plasma calprotectin determinations may contribute to the distinction between viral and bacterial infections and proper use of antibiotics. Sander *et al*[15] concluded that "low or normal L1 levels argue strongly against bacterial infection, while elevated L1 levels discriminate poorly between bacterial and non-infectious inflammatory or malignant disease."

Upregulation of S-100 protein CP-10 by endotoxin via distinct pathways has been demonstrated in murine cells in vitro.[126] It may be calculated that if most of the calprotectin in neutrophil granulocytes (about 5 pg/cell) in the blood of a healthy human individual (containing about 4×10^9 neutrophils per liter) is released in to the plasma, the concentration is expected to increase from 0.5 to about 20 mg/l.

Table 5 Clinical relevance of calprotectin reported in various disease conditions

Blood donors (plasma reference levels)[113]
Rheumatoid arthritis[12 13 22 94]
Sjøgrens syndrome[91]
Intraocular inflammatory conditions[95]
Systemic lupus erythematosus[96]
Cystic fibrosis[40 97]
Acute and chronic lung disease[98]
Lung carcinoma (squamous cells)[16]
Soft tissue tumor marker (non-specificity)[99 100]
Colorectal cancer[17 101 102]
Crohn's disease[19 103 104]
Ulcerative colitis[19 105 106]
Gastrointestinal mucosal inflammation[107 108]
Urinary stone[109]
Oral inflammatory mucosal disease[64]
CNS inflammatory disease (multiple sclerosis and acute encephalitis)[110]
Secondary CNS infections in HIV infected patients[18]
HIV infected patients[7 111]
Haematological patients[50]
Febrile conditions infectious and non-infectious[15]
Acute myocardial infarction[112]
Surgery[114-116]
Apheresis[117]

The references cited are representative reports or reviews, not a complete list of publications on each topic.

Table 6 Calprotectin concentrations in various body fluids

Body fluid	*References*	*Calprotectin concentrations**
Serum/plasma	Sander *et al*[15]	Infectious diseases Normal 0.1–0.6 Viral 0.1–1.4 Bacterial 0.6–11.0
	Müller *et al*[119]	HIV infection (25-75 percentiles) 1.2–9.4
	Dale[113]	Reference intervals Females 0.09–0.53 Males 0.12–0.66
	Berntzen *et al*[12-14]	Rheumatic disease Normal range 0.8–0.91 Rheumatoid arthritis 1–46 Osteoarthritis 0.5–0.8 Juvenile rheumatoid arthritis 2–24
	Haga *et al*[96]	Systemic lupus erythematosus, mean 3.6
	Semb *et al*[114]	Cardiopulmonary bypass operations Preoperative 0.3 Postoperative 5.2±1.3
	Garred *et al*[115]	Major surgery Preoperative baseline 0.5–0.9 Postoperative (4h) 7–15
	Lügering [103 106]	Crohn's disease Active 17±6 (MRP 8/14) Inactive 5±2 (MRP 8/14)
	Golden *et al*[97]	Cystic fibrosis children Controls 0.3–1.6 (median 0.7) Cystic fibrosis 0.4–26 (median 1.8)
	Ivanov *et al*[50]	Haematology Healthy blood donors 0.2 Leukaemia 0.4–13.3 (MRP 8/14)
Cerebrospinal fluid	Dunlop *et al*[18]	Normal range 0–37 µg/l HIV positive with infections 30–350 µg/l
Oral fluids	Brun *et al*[91]	Sjøgren's syndrome Stimulated whole saliva 23.6
	Cuida *et al*[121]	Healthy subjects Parotis saliva 3.2 Stimulated whole saliva 22.0 Mucosal transudate 40.9
	Müller *et al*[111]	HIV infected with or without oral candidiasis Parotis 0.06–0.41
Urine	Holt *et al*[120]	Infants and children (range) Controls 24 µg/l (5–650) Cystitis 182 µg/l (18–992) Pyelonephritis 1000 µg/ml (360–7000)
Faeces	Røseth *et al*[19]	Median (range) Healthy subjects 2 (0.5–8) Controls 10.5 (1.1–80) Crohn's disease 43 (8–2000) Ulcerative colitis 19.5 (2.4–866.4)
	Røseth *et al*[17]	Colorectal cancer 50.0 (4.5–950)
	Gilbert *et al*[102]	Healthy controls, range 0–9 Colorectal cancers (mean (SD)) Right side 55.1 (58.9) Left side 79.3 (58.2)
	Meling *et al*[108]	Mean (95% confidence intervals) Normal baseline 4.9 (1.5–15.6) NSAID treated 9.0 (6–27)
Synovial fluid	Berntzen *et al*[14]	Median (range) Rheumatoid arthritis 18 (2–375) Osteoarthritis 0.9 (0.2–2)
Empyema fluid (supernatant)	Santanagopalan *et al*[79]	Not quantitated. Calprotectin functional assay for *C albicans* growth inhibition

* Concentrations in cerebrospinal fluid and urine given as µg/l, all others given as mg/l.

Such values are found regularly in plasma from citrated blood stored for 14–21 days.

Increased plasma calprotectin concentrations are found regularly in HIV infected individuals, and a strong further increase in response to zidovudine treatment seems to predict a favourable prognosis.[119] Curiously, increased plasma calprotectin is associated with a poor chance of survival in patients with alcoholic liver disease irrespective of the degree of parenchymal liver damage.[127]

During surgery, complement activation can be demonstrated by increased concentrations of the terminal complement complex or C3b in plasma. Simultaneously, granulocyte lysosomal proteins (myeloperoxidase and lactoferrin) and calprotectin increase, but the latter remains elevated many hours longer than the former, even after the complement activation signals have returned to baseline.[116 128 129] Coronary sequestration of both granulocytes and calprotectin was demonstrated in the early reperfusion period after cardiac surgery and cold cardioplegic arrest.[114]

Recently, Arvesen *et al*[112] found that plasma calprotectin concentrations are increased in patients with coronary artery disease. Even patients with unstable angina or small (non-Q) myocardial infarctions had mean values about three times the upper reference limit. An interesting possibility is that the elevations may reflect an increased (inherited or acquired?) myelomonocytic response to even minor stimuli that in the long run may predispose to atherosclerosis.

Plasma calprotectin levels are increased in active rheumatic diseases; Berntzen *et al*[12-14] found 1–46 mg/l in rheumatoid arthritis

Table 7 Inflammation markers in meningococcal infections

	Calprotectin		*CRP*		*Leukocytes*		*LPS*		*IL-6*	
Clinical presentation	*mg/l*	*range*	*mg/l*	*range*	*x E9/l*	*range*	*ng/l*	*range*	*µg/l*	*range*
Fulminant sepsis*	13.8	10.5–119.9	85	19–225	7.1	2.2–33.3	2800	210–170000	550	55–2400
Meningitis*	6.8	2.9–51.0	137	24–328	22.6	3.8–30.9	<3	<3–210	0	0–297
Mild syst menin*	6.7	1.6–14.3	75	16–162	14.6	9.5–35.0	45	<3–600	3	0–102
Reference value†	0.7	0.3–1.6	≤10				<3		<0.05	

*Fulminant sepsis plasma (n=13) measured 12 h or less after onset of symptoms. Meningitis plasma (n=20) measured 24 h or less after onset. Mild systemic meningococcal (syst menin) disease plasma (n=6) measured at variable time intervals after onset of symptoms. Median value and range is given for each parameter.
† Reference values are from Golden *et al*[97] for calprotectin, Waage *et al*[124] for lipopolysaccharide (LPS) and interleukin (IL)-6, and Hjortdal *et al*[125] for C reactive protein (CRP). See also Brandtzæg and Kierulf.[23]

patients and 2–24 mg/l in juvenile rheumatoid arthritis, while Haga *et al*[96] reported a mean value of 3.6 mg/l in systemic lupus erythematosus patients compared with 1.05 mg/l in matched controls. In both rheumatoid arthritis and systemic lupus erythematosus, plasma calprotectin values appear to be an objective parameter for the assessment of disease activity and response to treatment. Furthermore, both plasma and synovial fluid calprotectin clearly distinguish rheumatoid from osteoarthritis.[14]

Plasma calprotectin concentrations are increased in endogenous posterior uveitis.[95] Patients may also have raised antineutrophilic cytoplasmatic antibodies (ANCA), but the ANCA titres did not correlate with calprotectin levels. The authors suggested the latter may be a sensitive indicator of disease activity for endogenous posterior uveitis. There seems to be a similar situation for cystic fibrosis.[97]

In a series of patients with newly diagnosed pulmonary cancers,[123] increased plasma calprotectin was found in 81%, while other disease indicating parameters lagged behind: CRP, 45%; orosomucoid, 48%; haptoglobulin, 59%; α_1 antitrypsin, 31%; alanine aminotransferase, 5%; aspartate aminotransferase, 0%; γ glutamyl transferase, 27%; alkaline phosphatase, 10%. Strikingly, about 15% of the patients had calprotectin concentrations (and other markers) close to or below the upper reference limit despite advanced, invasive disease. This raises the possibility that some tumours may release substances capable of inhibiting the emigration or activation of myelomonocytic cells. At least such cells would be expected to participate in the removal of dead tumour cells.

CALPROTECTIN IN CEREBROSPINAL FLUID

Limited data have been published, but calprotectin levels in CSF seem to distinguish between HIV encephalitis and opportunistic infections in the central nervous system in HIV infected patients.[18]

CALPROTECTIN IN ORAL FLUIDS

Concentrations between 3 and 40 mg/l have been measured in different oral secretions (table 6). Müller *et al*[111] found lower calprotectin concentrations in HIV infected patients who suffered from oral candidiasis compared with all HIV infected subjects, and with healthy controls.

CALPROTECTIN IN URINE

Increased calprotectin is found in patients with urinary tract infections, particularly in patients with renal involvement.[120] Calprotectin measurement has been suggested as a urinary marker of asthma.[130]

CALPROTECTIN AND GASTROINTESTINAL DISEASES

Røseth *et al*[19] developed a method for the determination of calprotectin in stool as an alternative to α_1 antitrypsin for the evaluation of disease activity in inflammatory bowel disease (IBD). It was, however, soon realised that increased faecal calprotectin is a marker of diseases of the gastrointestinal tract, including gastric cancer, colorectal adenoma or cancer, Crohn's disease, and ulcerative colitis.[17 19 101 105] The method depends on the preparation of a simple buffer extract of a small spot sample of stool, and quantitation of calprotectin by enzyme linked immunosorbent assay (ELISA). Only a fraction of the total calprotectin content in the stool sample is brought into solution, and most of it is found in high molecular size complexes (Røseth and Fagerhol, 1992, unpublished).

In recently diagnosed colorectal cancers, stool calprotectin concentrations above an upper reference limit of 10 mg/l were found in more than 90% of the patients.[19] In this study the value decreased below 10 mg/l after successful radical resection. No significant differences in calprotectin were found in cancers of Duke's stages A and B compared with C and D, which suggests that a positive calprotectin test may be found even in early cancers. This is supported by the finding of increased concentrations in more than 60% of patients with colorectal adenoma irrespective of size and location.[101] Again, calprotectin concentrations returned to normal after removal of the adenomas.[101] Although calprotectin is also synthesised by squamous epithelial cells, it is not found in epithelial cells of the bowel nor in colorectal cancer cells. The numbers of neutrophils, however, are increased significantly in the peritumoral stroma in colonic carcinomas.[131] The working hypothesis is that different types of pathological changes in the gastrointestinal tract cause increased permeability of the mucosa leading to increased migration of granulocytes and monocytes towards chemotactic substances in the gut lumen. An important factor for the high sensitivity of the faecal calprotectin test is the fact that this protein is remarkably resistant to proteolysis in the presence of calcium.[2] This may explain why the test has been found positive in more than 90% of patients with gastric cancer.[19]

It is notable that faecal calprotectin shedding may be caused by drugs[108] or other inflammatory reactions in the gastrointestinal system. Thus, extensive clinical studies are being conducted in this area, particularly aimed at confirming a negative predictive value for colorectal cancer.[17] Haemoglobin or faecal occult blood are used extensively as markers in screening for colorectal cancer. Calprotectin is a less variable parameter than haemoglobin in faecal samples from patients with colorectal cancers,[102] and although both parameters are elevated significantly, they do not correlate. Thus the mechanism of luminal calprotectin entry appears to be both different from and less erratic than bleeding.[102]

In patients with active IBD, faecal calprotectin concentrations above 100 mg/l are regularly found,[101] and a diagnosis of IBD should not be made in a symptomatic patient with a faecal calprotectin level below 10 mg/l. Highly significant correlations were found between this test and the IBD disease activity determined by endoscopy, histology or excretion of ^{111}indium labelled autologous granulocytes.[101 105] The latter method has been regarded as the "gold standard" but its use is limited by complexity, cost, and exposure of the patient to irradiation. The relatively simple faecal calprotectin test is an attractive alternative as it can be repeated at any time and, because of the stability of calprotectin, samples can be mailed by the patient to the laboratory. Treatment response in patients with Crohn's disease can be monitored by faecal calprotectin measurements.

CONCLUSION

The clinical relevance of calprotectin seems to be related to its physiological functions during homeostasis as well as in pathogenesis. These functions are intracellular as well as extracellular. Disease modulation during rheumatoid arthritis and possible antitumour effects of calprotectin may result from specific mechanisms other than antimicrobial activity. Calprotectin is a non-specific marker for activation of granulocytes and mononuclear phagocytes. It is released from these cells and is thus expected to be found in secretory and excretory products in different parts of the body.

Possible clinical applications

The diagnostic value of calprotectin has been emphasised in several publications (tables 5 and 6) that recommend calprotectin as an inflammatory disease marker. Extensive and systematic clinical documentation is still lacking, however, some larger studies are ongoing. RIA[33] and ELISA[1 5] have been established as immunoassays for calprotectin measurement in body fluids, and are also applied to stool samples.[17 19] Two commercial ELISA assays exist, with an analytical sensitivity at the ng/ml level. In the NycoCard (Nycomed, Oslo, Norway) immunoassay format[125 132] a prototype quantitative rapid test for calprotectin has been established with an analytical sensitivity of approximately 0.05 μg/ml. Documentation will increase as commercial calprotectin tests become easier and more widely available. Immunohistochemical staining for calprotectin may be a diagnostic tool in inflammatory skin diseases, malignant conditions, and a variety of other diseases.[3 133] Several commercial and non-commercial monoclonal antibodies are available (table 2).

Monitoring of disease activity and therapy may be relevant in inflammatory diseases such as rheumatoid arthritis, cystic fibrosis, Crohn's disease, asthma,[94 97 130] and others (tables 5 and 6).

Therapeutic use of calprotectin (or some of its structural elements) is a fascinating thought with its protective biological properties. Lehrer[32] calls it "an internal ointment that restricts the growth of *C albicans* on nearby skin." Could it be used as an external ointment? In terms of therapy could the molecule be used in rheumatic, inflammatory, infectious or malignant diseases?

Unpublished data

SYNTHESIS AND RELEASE OF CALPROTECTIN FROM HUMAN MONOCYTES IN VITRO

A series of experiments were performed immediately after the discovery of calprotectin, to gather data concerning the mechanisms involved in the release of calprotectin from monocytes. These data have not previously been published, nor have similar data been published by others. We have therefore included them in the present review.

Materials and methods

Human monocytes were isolated from healthy fasting donors as described previously[134 135] and cultured in RPMI 1640 (Gibco-Biocult, Paisley, Scotland) containing 20% inactivated fetal calf serum (Flow, Irvine, Scotland). The final cell preparations consisted of more than 95% monocytes demonstrated by differential counting, phagocytosis and staining for non-specific esterase.[136] The presence of calprotectin was determined by single radial diffusion or ELISA after incubation periods of up to 46 hours. The synthesis of calprotectin was studied by addition of ^{35}S-methionine to the medium, and radiolabelled calprotectin was determined by an immunoaffinity column with immobilised, monospecific rabbit anti-calprotectin antibodies.

The following compounds were added to monocyte cultures: immune complexes formed by incubating equivalent amounts of human serum albumin or transferrin (Sigma, St Louis, Missouri, USA) and corresponding antibodies (Dakopatts, Copenhagen, Denmark); aggregated human IgG (Gammaglobulin, Kabi, Stockholm, Sweden) prepared by heating a 1% solution in 0.15 M NaCl at 63°C for 12 minutes, and precipitation with Na_2SO_4 (67 g/l); cross-linked human immunoglobulins prepared by reacting IgG (10 g/l) with 0.5% glutaraldehyde in 0.1 M acetate buffer, pH 4.0 for one hour at room temperature, followed by dialysis against distilled water and phosphate buffered saline, pH 7.35; concanavalin A (ConA) and wheat germ agglutinin (WGA) (Sigma); phytohaemaglutinin (PHA) (Wellcome, Beckenham, Kent, UK); ionophores A 23187 and nigericin

Table 8 Release of calprotectin from human monocytes after 16 hours in vitro culture

Stimulant	*Concentration µg/ml*	*Serum* 20%*	*No. of cultures*	*Calprotectin release† (ng/ml)*
Lectins				
ConA	25	+	14	< 25
ConA	25	−	2	98
PHA	25	+	8	49
PHA	25	−	2	130
WGA	25	+	4	46
WGA	25	−	2	1050
Antigen/antibody complexes				
HSA/anti-HSA	10‡	+	8	665
HSA/anti-HSA	10‡	−	2	265
Transferrin/antitransferrin	2‡	+	6	440
Heat aggregated IgG (IgG-H)	700	+	2	15
Glutaraldehyde aggregated IgG (IgG-G)	700	+	2	25
HSA/anti-HSA + IgG-H	10‡ + 700	+	2	48
HSA/anti-HSA + IgG-H	10‡ + 250	+	2	52
HSA/anti-HSA + IgG-G	10‡ + 700	+	2	37
HSA/anti-HSA + IgG-G	10‡ + 250	+	2	49
Ionophores				
A 23187	0,25	+	14	165
A 23187	0,25	−	6	319
Nigericin	2,5	+	2	> 1000
Endotoxin	25	+	12	136
Phorbol ester				
TPA	1	+	2	2238

* Fetal calf serum inactivated at 56°C for 30 min.
† The release from unstimulated control cells was < 25 ng/ml.
‡ Amount of antigen present in the immune complex added.
HSA, human serum albumin.

Table 9 Release of lysozyme and β glucuronidase from stimulated human monocytes in vitro

*Stimulant**	*Lysozyme % of unstimulated control (SEM)*	*Cultures (n)*	*β glucuronidase % of unstimulated control (SEM)*	*Cultures (n)*
Immune complexes	695 (189)	12	610 (79)	8
Endotoxin	138 (45)	10	107 (4)	8
A 23187	435 (251)	12	198 (68)	10
Lectins	92 (8)	16	97 (21)	14
Nigericin	257	2	356	2
TPA	664 (114)	10	425 (70)	10

* The final concentrations of stimulants were as given in table 8.

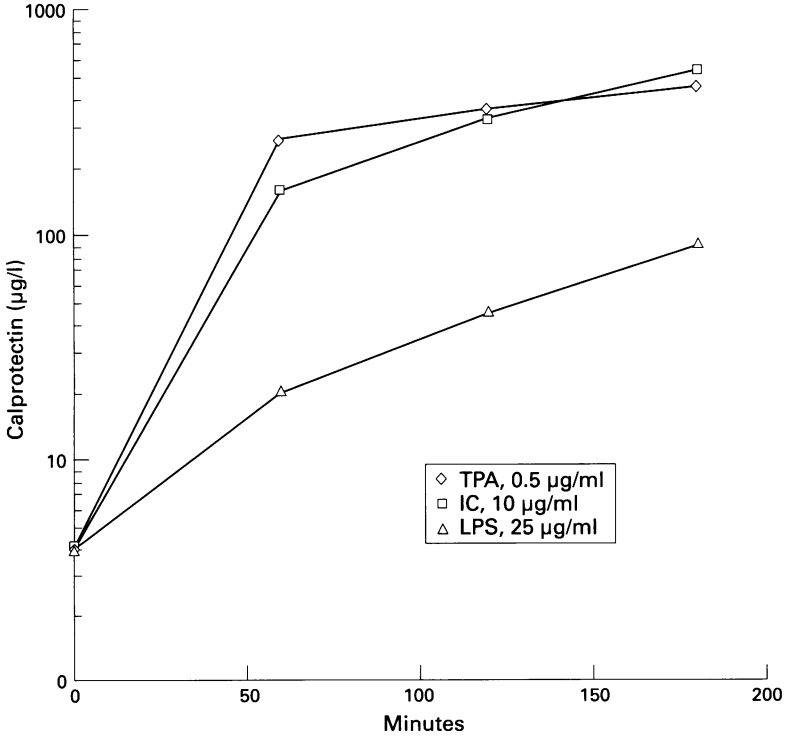

Figure 1 Time course for release of calprotectin from human monocytes in vitro. TPA, 12-O-tetradecanoylphorbol-13-acetate; LPS, endotoxin; IC, immune complexes. The final concentrations in µg/ml of the added substances are indicated.

(Lilly Research Corporation, Indianapolis, Indiana, USA); endotoxins *E coli* 0111:B4 and *E coli* 055:B5 (Difco, Detroit, Michigan, USA); actinomycin D (Serva, Heidelberg, Germany); 12-O-tetradecanoylphorbol-13-acetate (TPA) (PL Biochemicals, Milwakee, Wisconsin, USA); cycloheximide, cytochalasin B, and prostaglandin E_1 (PGE_1) (Sigma).

Lactate dehydrogenase, lysozyme, and β glucoronidase activities were determined in culture supernatants and cell homogenates.[137-139]

Results

Freshly prepared monocytes contained 2.5 ± 0.7 pg calprotectin per cell, and after incubation for 9–10 hours the content was 2.3 ± 0.2 pg/cell.

Various agents known to interact with and activate[140] monocytes were added to monocyte cultures. None of these, except TPA at 1 µg/ml caused cytotoxicity (increased trypan blue uptake) within the time period studied. As shown in table 8, very small amounts of calprotectin were released from monocytes cultured for 16 hours in the presence of lectins and 20% inactivated fetal calf serum. In the absence of serum slightly higher amounts (but less than 1% of total cellular calprotectin content) were released after ConA or PHA stimulation, while WGA caused a noticeably higher release. No release of lactate dehydrogenase or β glucuronidase, and no increased lysozyme release was seen in lectin stimulated cultures (table 9). Immune complexes led to a marked release of calprotectin over two to three hours (fig 1), and about 8% of the calculated total cellular calprotectin was released. This release was two to threefold higher in the presence of fetal calf serum (table 8). Native serum was 25% more active than inactivated serum, and native serum alone promoted the release of calprotectin in the absence of immune complexes. Addition of immune complexes induced a marked release of both β glucuronidase and lysozyme (table 9), but essentially no release of lactate dehydrogenase.

Phorbol ester (TPA) at 500 ng/ml caused a quite rapid and marked release of calprotectin (about 7% of total cellular content), but no signs of cytotoxicity in three hours (fig 1). Release of the total intracellular calprotectin was seen when a cytotoxic dose of TPA (1 µg/ml) was applied for 14 hours (table 8). The divalent ionophore A23187 caused a moderate release of calprotectin, and the monovalent ionophore nigericin a substantial release (table 8). Both ionophores increased moderately the release of β glucuronidase and lysozyme (table 9).

Endotoxin induced a moderate but distinct release of calprotectin (table 8), but no significant increase in lysozyme or β glucuronidase secretion (table 9). A very small amount (less than 2% of total cellular content) was released during the first three hours (fig 1).

To learn more about the mechanism of calprotectin release, addition of several other compounds were tested. Prostaglandin E_1 (10 µM) did not influence the release of calprotectin during incubation with TPA. The

Table 10 Release of calprotectin, lysozyme, and β glucuronidase from stimulated human monocytes in vitro, with potentially interfering substances added

*Stimulant**	*Additions†*	*Calprotectin‡*	*Lysozyme‡*	*β glucuronidase‡*
Immune complexes	Dexamethasone	101 (7)	84 (7)	87 (35)
	Promethazine	98	131	134
	Indomethacin	81 (12)	100 (5)	115 (8)
Endotoxin	Dexamethasone	44 (6)	99 (15)	141
	Promethazine	248 (70)	1250	597 (260)
A 23187	Dexamethasone	122	93 (7)	18
	Promethazine	311	452	426
	Indomethacin	196 (12)		
Lectins	Dexamethasone	95 (25)	101 (18)	58 (15)
	Indomethacin			230

* The final concentrations of stimulants were as given in table 8.
† Final concentrations: dexamethasone, 10 μg/ml; promethazine and indomethacin; 0.1 mM.
‡ Each value is the mean of 2–8 cultures, and calculated as % of stimulated control (SEM).

prostaglandin synthesis inhibitors indomethacin and acetylsalicylic acid had no effect on the release caused by immune complexes or lectins (table 10). Release induced by A23187 was enchanced twofold by indomethacin but not by acetylsalicylic acid. As indomethacin has other effects besides blocking prostaglandin synthesis,[141] our tentative conclusion is that products of the prostaglandin pathway are not involved in calprotectin secretion. The antihistamine promethazine enhanced the release of calprotectin induced by endotoxin, A23187 or TPA (data not shown), whereas the release induced by immune complexes was unchanged (table 10). Similarly, both lysozyme and β glucuronidase release was enchanced by addition of promethazine to A23187 or endotoxins, but not to immune complex stimulated monocytes (table 9). Dexamethasone inhibited calprotectin release induced by endotoxins but not that induced by A23187, lectins or immune complexes. This drug had no effect on the lysozyme release, but the release of β glucuronidase induced by A23187 and lectins was inhibited. Cytochalasin B (10 μg/ml), which interferes with actin polymerisation, had no effect on calprotectin release induced by endotoxin, but a twofold enhancing effect in the presence of PHA.

Dexamethasone (10 μg/ml), dibutyryl cAMP (1 mM), cAMP (1 mM), verapamil (25 μg/ml), acetylsalicylic acid (0.1 mM), and purified protein derivative of Bacillus Calmette-Guerin (PPD) had no effect of calprotectin release from unstimulated cells.

Discussion

The release of calprotectin from isolated monocytes can result from general cytotoxicity. If sufficiently high doses are used—for example, TPA at 1 μg/ml, the total calprotectin pool may be released. However, partial release was observed without concomitant lactate dehydrogenase release or trypan blue uptake. This suggests that either calprotectin release is a very sensitive indicator of cytotoxicity or the release is caused by other mechanisms.

There was no definite evidence for involvement of the prostaglandin system in calprotectin release, and the fraction released during three to four hours never exceeded 8% of the total cellular pool.

The agents that promoted lysosomal enzyme release most (immune complexes, TPA, ionophores) also increased the calprotectin release, although not exactly in the same proportion. A correlation was observed between lysozyme and calprotectin release. The effects of dexamethasone served to distinguish these processes in that it reduced calprotectin release while leaving lysozyme and β glucuronidase release unchanged in response to endotoxin. In the presence of lectins or A23187 the opposite was found: dexamethasone reduced β glucuronidase release whereas calprotectin and lysozyme release were unchanged. It is therefore likely that calprotectin release is different from the lysozyme and lysosomal release processes. Clearly, calprotectin release can be caused by a number of membrane perturbing agents, and calprotectin is either derived from the plasma membrane or from an intracellular compartment by some special release process.[142]

Anti-calprotectin antibodies added to human monocytes in culture induced the synthesis of the protein component of thromboplastin,[140] which suggests that calprotectin is at least in part located in the plasma membrane, as observed by Dale *et al*[75] and Guignard *et al*.[88]

Conclusions

Calprotectin is fascinating because it holds unrevealed secrets. We know that calprotectin is an important granulocyte marker and a multifunctional regulatory protein in inflammatory processes. There is more work to do in order to understand fully basic mechanisms such as quaternary structure, complex assembly, molecular functions, and biological effect mechanisms.

Much has been reported regarding the clinical relevance and diagnostic potential of calprotectin and its subunits, and larger clinical studies are continuing. Further work may gather speed as commercial kits for calprotectin measurement become more readily available. Interesting therapeutic concepts may be found for calprotectin, as the relation of the structure and function of the molecule are better understood.

In the meantime we will do as Lehrer[32] recommended in 1993: "Stay tuned. Calprotectin, whatever it is, could be interesting."

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