EXTENDED REPORTS

Altered Ca^{2+} signalling in human neutrophils from inflammatory sites

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Abstract

Objectives—To determine whether the intracellular store release of Ca^{2+} in neutrophils from patients with rheumatoid arthritis, other joint disease and active leg ulceration was different from normal neutrophils.

Methods—The release into the cytosol of Ca^{2+} from stores within individual neutrophils was determined using ratiometric imaging of fura2. The size of the elevated Ca^{2+} 'cloud' and its concentration were quantified in neutrophils from the circulation of patients with rheumatoid arthritis, other joint diseases, and leg ulcers and from the joints of those with joint disease.

Results—In neutrophils isolated from both the synovial fluid of patients with rheumatoid arthritis and other joint conditions, and also arising from leg ulcers, the amount of the cell cytosol occupied by elevated Ca^{2+} was significantly increased compared with neutrophils from healthy subjects; for neutrophils from rheumatoid, non-rheumatoid joints and leg ulcers p values were 0.0006, <0.0001, 0.016 respectively (Student's t test). There was also a significant increase in Ca^{2+} release from circulating neutrophils from patients with rheumatoid arthritis ($p = 0.09$), but not in circulating neutrophils from patients with leg ulcers or non-rheumatoid joint conditions.

Conclusions—It is proposed that the increased release of free Ca^{2+} into the cytosol of neutrophil at inflammatory sites results in increased oxidase activation.

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Neutrophils constitute up to 90% of the inflammatory infiltrate in inflammatory based diseases, such as rheumatoid arthritis.¹ Their persistence at the inflammatory site is accompanied by severe tissue damage. This is a result of activation of non-mitochondrial neutrophil oxidase and the secretion of hydrolytic and degradative enzymes, both cellular events being triggered by an increase in cytosolic free Ca^{2+} concentration.^{2,3} This elevation in cytosolic free Ca^{2+} results from both an influx of Ca^{2+} from the extracellular medium and release of Ca^{2+} from stores within the cell.^{4,5} Release of Ca^{2+} from the store can be seen in the absence of extracellular Ca^{2+} ,^{4,5}

or by pharmacologically blocking transmembrane influx.⁶ Under these conditions, we have observed 'clouds' of elevated Ca^{2+} which result from the release of Ca^{2+} from the store, the ' Ca^{2+} cloud' being larger in neutrophils from the synovial fluid of patients with rheumatoid arthritis than from circulating neutrophils from healthy subjects.⁷ The question arises as to whether this increased Ca^{2+} store release is unique to rheumatoid arthritis or whether similar changes in Ca^{2+} storage occur in neutrophils in other inflammatory conditions. We have therefore compared the intracellular store release of Ca^{2+} in neutrophils from patients with rheumatoid arthritis, psoriasis, gout, ankylosings spondylitis, non-specific synovitis, reactive arthritis and active leg ulceration. We report the increased release of Ca^{2+} from the store in all these inflammatory conditions. Only in circulating neutrophils from patients with rheumatoid arthritis was the increased Ca^{2+} store also detectable.

Materials and methods

PATIENTS

Synovial fluid was taken from the articular cavity of patients with rheumatoid arthritis, sero negative arthritis, gout, psoriasis, ankylosing spondylitis, non-specific synovitis and reactive arthritis (table 1) by needle puncture. Neutrophils were isolated from EDTA treated synovial fluid as previously described.⁷

Venous samples were obtained from patients with either active leg ulceration or with skin changes of lipodermatosclerosis secondary to venous disease. Venous incompetence was determined using Doppler ultrasound and significant arterial impairment excluded by measurement of the ankle brachial pressure

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Table 1 Patient details

Patient group	Sex		Total	Age range Mean (SE)
	Male	Female		
RA sero +ve	4	6*	10	51 (4)
RA sero -ve	0	2	2	31 (2)
Gout	2	1	3	60 (8)
Psoriasis	2	0	2	51 (7)
Ankylosing spondylitis	1	0	1	58
Reactive arthritis	1	0	1	32
Non-RA joint disease	6	3	9	46.4 (7)
Leg ulcers	3	4†	7	74 (3)
Normal control	8	4	12	31 (7)

*Two patients had been previously treated by drug therapy: one with penicillamine and one with sulphasalazine.

†One patient had been previously treated with trental.

Figure 1 Localised 'clouds' of elevated Ca^{2+} in individual neutrophils. Pseudo 3d-representations of cytosolic free Ca^{2+} concentration, where the x-y plane is the area occupied by the cell and the z-plane is the cytosolic free Ca^{2+} concentration for (A) a normal circulating neutrophil, (B) a circulating neutrophil from a leg ulcer, (C) a synovial fluid neutrophil from a patient with rheumatoid arthritis, and (D) a synovial fluid neutrophil from a patient with sero negative joint disease. In all cases the cells were stimulated with f-met-leu-phe ($1 \mu\text{M}$) in the absence of extracellular Ca^{2+} (1 mM EGTA). The percentage area of the cloud calculated as that exceeding the 250 nM cytosolic free Ca^{2+} concentration level is also shown. The examples shown are representative of the groups shown.

index. One sample of blood was taken from the arm of the patient and a second from a tributary of the saphenous vein above the affected ankle. The second sample was obtained after the patient had been resting with the affected leg dependent for 30 minutes followed by elevating the leg to the horizontal for a further 5 minutes. These times were chosen to coincide with the efflux of entrapped neutrophils from the leg.^{8,9} Neutrophils were isolated from heparinised peripheral blood of all patients and healthy volunteers as previously described.^{4,5,7}

Neutrophils isolated in this study did not have elevated cytosolic free Ca^{2+} concentration or oxidase activity. Although all samples were treated identically, the possibility that neutrophils from different sources may be affected differently by isolation, could not be excluded.

IMAGING RELEASE OF Ca^{2+} FROM INTRACELLULAR STORES

Neutrophils ($2 \cdot 10^7$ cells/ml) were loaded with fura-2⁹ and ratio imaging of neutrophils adherent to glass coverslips was performed as previously described.^{4,5,10,11} Neutrophils were stimulated with f-met-leu-phe ($1 \mu\text{M}$) in the absence of extracellular Ca^{2+} (medium also containing 1 mM EGTA). The 'cloud' of elevated Ca^{2+} was defined as being above a threshold concentration of 250 nM .^{4,5} The area of the neutrophil occupied by the 'cloud' and the mean Ca^{2+} concentration in the 'cloud' was determined by pixel-by-pixel analysis of the image (fig 1).

Results

' Ca^{2+} CLOUDS' IN NEUTROPHILS FROM JOINT FLUID

In the absence of extracellular Ca^{2+} , a transient and localised cytosolic free Ca^{2+} rise in neutrophils from all sources was observed when stimulated by f-met-leu-phe ($1 \mu\text{M}$), as a result of Ca^{2+} store release (fig 1).

Elevation of cytosolic free Ca^{2+} concentration from stores was detectable in 20% to 80% of neutrophils examined from rheumatoid disease and sero-negative disease in response to f-met-leu-phe. The heterogeneity of responsiveness has been reported previously⁴ and results from both the presence of non-responding but viable cells¹² and an inability to detect small localised changes non-confocally. The localised elevation of Ca^{2+} in these cells was transient, occurring between 7 s and 14 s after addition of the stimulus, and returning to resting Ca^{2+} levels by 50 s. Although the peak Ca^{2+} concentrations in the cloud during this time was similar in neutrophils from all sources examined, the maximum area occupied by the elevated Ca^{2+} cloud was larger in cells from inflammatory joints than from the circulation of healthy controls (table 2). The area of the Ca^{2+} cloud was larger in neutrophils isolated from both the synovial fluid of patients with rheumatoid arthritis ($p = 0.0006$) and other non-rheumatoid inflammatory joint conditions ($p < 0.0001$) compared with neutrophils from healthy subjects (figure 2A, B and C). One anomaly was the size of the elevated Ca^{2+} cloud in neutrophils from two patients with reactive arthritis, which was smaller than from

Table 2 Ca^{2+} Release from intracellular stores

Patient group	Size of Ca^{2+} cloud (% cell area) mean (SEM)	median (Q1–Q3)	Mean $[\text{Ca}]_i$ in cloud (nM) mean (SEM)	Number of responding cells fraction*
<i>Synovial fluid neutrophils</i>				
Sero –ve RA	35 (13)	16.5 (4.2–76.5)	438 (47)	8/17
Ankylosing spondylitis	75	74	575	1/2
Gout	58 (6)	56 (50–70)	428 (61)	4/8
Psoriasis	52 (25)	52	329 (99)	2/11
Reactive A.	10 (2)	10	411 (11)	2/3
Synovitis	31 (11)	23 (17–52)	418 (49)	3/6
RA sero +ve	52 (6)	48 (21–80)	532 (30)	22/36
<i>Blood neutrophils</i>				
Ulcer (leg)	30 (7)	25 (12.25–48)	507 (50)	18/26
Ulcer (arm)	12 (3)	9.5 (3.3–20.3)	465 (44)	13/22
Sero –ve RA	4 (2)	4	317 (5)	2/8
Other	18 (6)	15 (2.5–22)	414 (39)	7/10
RA sero +ve	40 (9)	34 (10–70)	300 (39)	11/22
Control	18 (4)	7 (2–35)	386 (19)	45/85

*The fraction expresses the number of cells giving a measurable Ca^{2+} response divided by the total number of cells observed.

neutrophils from other joint conditions, and was not significantly different from neutrophils of the circulation of healthy subjects.

‘ Ca^{2+} CLOUDS’ IN NEUTROPHILS FROM LEG ULCERS

Neutrophils isolated from blood taken from near leg ulcers also had significantly increased release of Ca^{2+} ($p < 0.0001$) compared with healthy circulating neutrophils (table 2). The area of the Ca^{2+} cloud in these neutrophils isolated from blood taken from the region of the ulcer was also significantly larger ($p = 0.016$) than in neutrophils isolated from peripheral blood from the same patients (figure 2). Neutrophils from the site of the ulcer had clouds which occupied up to 88% of the cell, whereas the cloud size in circulating cells was not above 18% of the cell (fig 2). The percentage area of the Ca^{2+} cloud in neutrophils from the circulating blood of patients with leg ulcer, or non rheumatoid arthritis joint disease was not significantly different from the cloud size in neutrophils from blood from healthy subjects. However, the size of the cloud in neutrophils from the circulation of patients with rheumatoid arthritis, though not as large as in neutrophils from the joint itself, was significantly larger than neutrophils from the circulation of healthy subjects ($p = 0.09$) or patients with leg ulcers ($p = 0.036$) (fig 2, table 3).

Discussion

The data presented in this paper demonstrate that Ca^{2+} signalling in neutrophils from inflammatory sites is different from normal. This modification occurs in neutrophils from both joints of patients with rheumatoid arthritis and non-rheumatoid conditions and also from another inflammatory site, leg ulcers. The modification in Ca^{2+} handling resulted in

Table 3 Synopsis of data for area occupied by Ca^{2+} cloud

Synovial fluid	Blood	Ulcer blood
	Normal = 18 (4)%	
Non-RA = 41 (7)%	Non-RA = 16 (4)%	Circulating = 12 (3)%
RA = 52 (6)%	RA = 40 (9)%	Ulcer = 30 (7)%

Figure 2 Comparison of the sizes of the Ca^{2+} 'cloud'. The Ca^{2+} cloud sizes, determined as shown in figure 1, are shown (A) for circulating neutrophils from the arm (UBL) and leg (ULBL) of patients with leg ulcers; (B) for circulating neutrophils of patients with sero-positive (■) and sero-negative (▲) rheumatoid arthritis (RABL) and for neutrophils from the synovial fluid of the same patients (RASF); and (C) for circulating neutrophils from patients with non-rheumatoid arthritic joint disease (Non-RABL) and from neutrophils from the synovial fluid of the same patients (Non-RASF). The symbols for the non-rheumatoid arthritic diseases are as follows; gout (▼), psoriasis (■), ankylosing spondylitis (▲) reactive arthritis (★) and non-specific synovitis (◆). In both graphs the mean (SEM) of the population is also shown.

a less restricted area of elevated Ca^{2+} arising from intracellular Ca^{2+} store release. This

study also found that, whereas Ca^{2+} storage and release in neutrophils from the circulation of patients with non-rheumatoid joint disease or leg ulcers was not different from healthy circulating neutrophils, in rheumatoid arthritis the circulating cells also showed a significant increase in the area of cell cytosol occupied by Ca^{2+} released from the Ca^{2+} store.

This study therefore raises two main questions. Firstly, are the changes in Ca^{2+} signalling in neutrophils a cause or consequence of inflammation? As neutrophils from the synovial fluid of non-erosive joints have similarly elevated Ca^{2+} clouds to those from rheumatoid arthritic joints, it is unlikely that this aberration in Ca^{2+} handling alone is responsible for tissue damage. We have, however, shown previously that the localised elevation in cytosolic free Ca^{2+} concentration in response to f-met-leu-phe, triggers a localised activation of the oxidase in neutrophils.⁵ The possibility thus exists that a combination of aberrant Ca^{2+} handling with a stimulus which releases store Ca^{2+} would result in pathogenic oxidase activation of joint neutrophils. We have recently shown that the initial contact of neutrophils with soluble immune complexes, which is present in the joints of patients with rheumatoid arthritis,¹³ result in the release of Ca^{2+} from stores before the accompanying transmembrane Ca^{2+} influx.¹⁴ The response to this stimulus thus relies on stored Ca^{2+} for triggering the response and may be exaggerated in neutrophils with larger Ca^{2+} stores. Thus the combination of neutrophils with increased Ca^{2+} storage with local stimulatory factors which utilise this Ca^{2+} , would provide an explanation for the aberrant behaviour of neutrophils at these sites.

The second question arises as to the mechanism of increased size of elevated Ca^{2+} 'cloud' after release from the Ca^{2+} store both in joint and circulating rheumatoid arthritis neutrophils? Changes in cytosolic free Ca^{2+} handling may occur as a result of the processes of extra-vascularisation. It is known, for example, that engagement of neutrophil integrins^{15 16} and chemotactic factors elevate cytosolic free Ca^{2+} concentration^{17 18} and may thus affect subsequent Ca^{2+} signalling. The alteration in Ca^{2+} signalling in circulating neutrophils in rheumatoid arthritis may result from circulating factors which modify Ca^{2+} handling or be the result of neutrophils returning to the circulation with aberrant Ca^{2+} signalling, as can occur in venous disease. Another possibility is that chronic inflammation stimulates the bone marrow to produce a population of neutrophils which have Ca^{2+} signalling properties different from normal.

The results in this paper report the first comparative study of Ca^{2+} signalling in rheumatoid arthritis using Ca^{2+} imaging of individual neutrophils. We propose that the changes in Ca^{2+} signalling identified here result from alterations in Ca^{2+} binding proteins, either within the cytosol or within the Ca^{2+} store. It will be necessary to determine whether alterations in the expression of calreticulum or other cellular calcium binding proteins underlies the phenomenon reported here.

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