Aberrant Integrin (CR4; \(\alpha_N\beta_2\); CD11c/CD18) Oscillations on Neutrophils in a Mild Form of Pyoderma Gangrenosum

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We have previously shown that the \(\beta_2\) integrins CR3 and CR4 physically and functionally interact with urokinase receptors (uPAR) on neutrophil plasma membranes in an oscillatory fashion. In this study we have analyzed neutrophils from patient SC, a 34 y old African American female, with aberrant skin window results and recurrent perianal abscesses and pretibial lesions diagnosed as pyoderma gangrenosum. Although untreated migrating normal neutrophils exhibited 20 s sinusoidal oscillations in CR4–uPAR proximity, neutrophils from SC demonstrated a faster oscillation (10 s) in the form of a flyback sawtooth wave. This waveform mimicked that observed for normal neutrophils treated with subsaturating doses of the kinase inhibitors staurosporine, genistein, and erbastin. As \(\beta_2\) integrins are regulated by phosphorylation, we tested the hypothesis that the aberrant CR4–uPAR proximity oscillations seen in SC’s neutrophils are due to defective kinase activity that might be balanced by a decrease in phosphatase activity. When SC’s cells are exposed to subsaturating concentrations of the phosphatase inhibitor pervanadate, this caused the CR4–uPAR oscillations to become sinusoidal in shape with a 20 s period, as seen in normal migrating neutrophils. Although SC’s neutrophils were deficient in spontaneous and N-formyl-methionyl-leucyl-phenylalanine-induced polarization, 0.5 \(\mu\)M pervanadate returned cell polarization to nearly normal levels, thus paralleling the acquisition of normal receptor interactions. Inasmuch as SC’s cellular phenotype is mimicked by kinase inhibitors and corrected by phosphatase inhibitors, we suggest that a mutation(s) affecting the kinetics of intracellular signaling enzymes, but not blocking the pathway per se, may be responsible for this clinical state.


Pyoderma gangrenosum is a painful, chronic, and ulcerating skin condition. It is diagnosed on the basis of clinical features, as there are no characteristic laboratory or histopathologic features (Holt et al., 1980). Although adult patients generally have an associated systemic abnormality (arthritis, inflammatory bowel disease, etc.), pediatric patients may present with disease at sites of trauma. The pathogenesis of pyoderma gangrenosum is unknown because in part to its heterogeneity; however, analysis of certain pediatric patients suggests aberrations in immunologic function (Delescluse et al., 1972; Lazarus et al., 1972; Brandt et al., 1977; Clayton, 1977; Holt et al., 1980). Aberrations in neutrophil function, including motility and phagocytosis, have been reported (Miller and Dooley, 1973; Hickman, 1983; Malech and Gallin, 1987; Teitel, 1996; Adachi et al., 1998). Steroids and methotrexate have been reported to be useful in the clinical management of disease (Teitel, 1996); however, because the cellular and molecular nature of pyoderma gangrenosum is not known, there is no rational basis for therapy. Recently, we have described a pediatric onset case of pyoderma gangrenosum with an associated defect in neutrophil motility; this motile deficiency was, in turn, traced to aberrent leukocyte integrin clustering and metabolic oscillations. Thus, we have suggested that a subset of the pediatric-onset pyoderma gangrenosum patients may be categorized at a dynamical disease in neutrophil regulation and have begun rational changes in patient therapy (Adachi et al., 1998).

Several years ago we were the first to identify lateral interactions between leukocyte integrins and glycosylphosphatidylinositol-linked membrane proteins (e.g., Zhou et al., 1993; Sehgal et al., 1993). Using several experimental techniques, we and others have confirmed and extended these findings on the inter-receptor interactions of leukocyte integrins and other membrane proteins (e.g., Xue et al., 1994, 1997; Kraus et al., 1994; Cao et al., 1995; Poo et al., 1995; Bohuslav et al., 1995; Wei et al., 1996; Sitrin et al., 1996; Annenkow et al., 1996; Worth et al., 1996; Kindzelskii et al., 1996, 1997). A central feature of cell motility is the rhythmic interactions of integrins and the substrate (Springer, 1990). Recently, parallel results indicating rhythmic inter-receptor interactions between CR4 and uPAR have been observed (Kindzelskii et al., 1997). In this study, a patient with a mild form of pediatric-onset pyoderma gangrenosum with aberrant skin-window findings was evaluated. Neutrophil receptor properties of this patient reveal the dynamical character of this disorder, as the defect is associated with the kinetics of inter-receptor interactions in neutrophil membranes.

MATERIALS AND METHODS

Materials Genistein and FMLP (N-formyl-methionyl-leucyl-phenylalanine) were obtained from Sigma (St. Louis, MO). Staurosporine and erbastin were purchased from Calbiochem (La Jolla, CA).

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Abbreviations: FMLP, N-formyl-methionyl-leucyl-phenylalanine; RET, resonance energy transfer.

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Preparation of neutrophils Human neutrophils were purified from the peripheral blood of normal donors (American Red Cross, Detroit, MI) or patient SC. All procedures were approved by the Internal Review Board with informed consent from participants. Heparinized whole blood was subjected to Histopaque (Sigma) step-density gradient centrifugation (Cao et al., 1995). The remaining red blood cells were depleted by hypotonic lysis. The cell preparation was ≈95% neutrophils. The purified neutrophils were typically 95% viable, as assessed by trypan blue exclusion.

Immunofluorescence labeling Mouse monochonal antibodies to CR3 (anti-Mo1, clone 44), the urokinase-type plasminogen activator receptor (clone 3B10), CR4, and FcγRIIIIB were obtained as previously described (Zhou et al., 1993; Kindzelskii et al., 1997). All experiments used Fab or Fab′2 fragments of antibodies. Antibody fragments were conjugated with fluorescein or rhodamine as described (Kindzelskii et al., 1997).

Fluorescence microscopy Optical microscopy of labeled cells was carried out using a Zeiss axiovert microscope (Carl Zeiss, New York, NY) attached to a Percepts (Knoxville, TN) image processing system. A Zeiss temperature stage was used to hold sample temperature at 37°C in all experiments. Differential interference contrast microscopy was performed using Zeiss polarizers and analyzers. The optical filters and data handling were as previously described for differential interference contrast and epifluorescence microscopy (Zhou et al., 1993). Fluorescence images were collected using an intensified charge-coupled device camera (Hamamatsu model XC–77). Resonance energy transfer (RET) microscopy and quantitative RET measurements were performed as described (Kindzelskii et al., 1997).

For quantitative analysis of cell shape, the total number of cells and the total number of polarized cells, which are triangular in shape, were determined in randomly selected but nonoverlapping fields. The total number of cells counted for each condition varied from 250 to 500 cells per experimental sample. In some cases 10^7 M FMLP was added to stimulate cells.

Case report SC is a 34 y old African American female who initially presented to the Children's Hospital of Michigan in 1972 with a perineal abscess at the age of 13. On this initial visit, no treatment was initiated and the abscesses healed spontaneously.

In 1975, SC presented to the Children's Hospital of Michigan again with a perineal abscess with a yellowish discharge. During this hospital stay, cultures grew out Escherichia coli and Streptococcus, SC, during this episode, also developed multiple lesions throughout her lower extremities. Moreover, pain and swelling developed in both knees, ankles, and the right wrist. The patient could not perform her normal daily activities such as riding a bicycle or walking. The erythrocyte sedimentation rate was 53 mm per h. X-rays of the knees showed bilateral effusions; a right knee arthrocentsis was performed that showed acute granulomatous inflammation. She was treated with oxacillin, prostaphlin, betadine dressings, local wound care, and scheduled aspirin. The patient's symptoms subsided and she was discharged.

During 1975 the patient presented on three additional occasions to the Children's Hospital of Michigan with perineal abscesses as well as perinaeal skin lesions throughout the legs and the hands (Fig 1). Cultures of the abscesses during these episodes were sterile. Further diagnostic evaluation was remarkable for an increase in IgA, a defective chemotaxis of neutrophils, and an absence of mononuclear and macrophages in the skin window test. The patient was again routinely managed with aggressive local wound care, sitz baths, and betadine dressings.

Since 1975, SC has continued to experience frequent cutaneous lesions consistent with the diagnosis of pyoderma gangrenosum. Various medical treatments including multiple surgeries including skin grafting in the axills and perineum have been performed with little lasting success. More recently, SC has also had a history of nondeforming arthritis.

RESULTS Neutrophil receptor oscillations in pyoderma gangrenosum resemble kinase-inhibited normal cells On the basis of the patient's clinical disease, the clinical laboratory findings, and our previous observations concerning pyoderma gangrenosum (Adachi et al., 1998), we tested the hypothesis that other forms of pyoderma gangrenosum display defects in biochemical oscillators of neutrophils. Normal neutrophils display sinusoidal oscillations of CR4-to-uPAR proximity (Fig 2a) and NAD(P)H autofluorescence (Fig 3c) with periods of ≈20 s, as we have previously noted (Kindzelskii et al., 1997, 1998). When neutrophils are exposed to the chemotactic factor FMLP, the oscillation period was reduced to about 10 s (Fig 2b) (Kindzelskii et al., 1997). Importantly, when normal neutrophils are treated with low sub saturating doses of kinase inhibitors to only partially block enzyme action, a nonsinusoidal oscillation of increased frequency was observed (Fig 2c–e). At high doses, kinase inhibitors block receptor oscillations and cell motility in vitro. We therefore titrated these reagents to obtain the highest doses that did not block cell polarization. Thus, we employed 0.05 μM staurosporine (Fig 2e), 150 μg genistein per ml (Fig 2d), and 20 μg erbstatin per ml (Fig 2e). Under all of these conditions CR4-uPAR proximity oscillations were observed to be in the form of a flyback sawtooth waveform of increased frequency. Therefore, several kinase inhibitors in addition to staurosporine (Kindzelskii et al., 1997) affect the frequency and waveform of CR4-uPAR oscillations.

When SC's cells were tested using immunofluorescence microscopy, no integrin clusters were observed at 37°C (data not shown). This contrasts sharply with our previous study of a patient with more severe disease (Adachi et al., 1998). Also in contrast to this previous study, SC's cells exhibited sinusoidal NAD(P)H oscillations (Fig 3f); however, we discovered that SC's neutrophils exhibited a flyback sawtooth waveform in CR4-uPAR proximity (Fig 3d) with a period of 10.6 ± 1.8 s (Table 1). This waveform was similar to that previously observed for staurosporin-treated normal cells; i.e., her cells behaved as though they were exposed to a kinase inhibitor. Thus, SC's neutrophils exhibit a defect in the kinetics of CR4-uPAR oscillations, not in their presence or absence – thus underscoring the dynamics of the defect at the molecular level.

Pervanadate restores normal receptor oscillations We have previously suggested that the oscillatory CR4-uPAR waveform is a reflection of the oscillatory signal transduction apparatus (Kindzelskii et al., 1997). Importantly, when normal neutrophils are treated with low sub saturating doses of kinase inhibitors to only partially block enzyme action, a nonsinusoidal oscillation of increased frequency was observed (Fig 2c–e). At high doses, kinase inhibitors block receptor oscillations and cell motility in vitro. We therefore titrated these reagents to obtain the highest doses that did not block cell polarization. Thus, we employed 0.05 μM staurosporine (Fig 2e), 150 μg genistein per ml (Fig 2d), and 20 μg erbstatin per ml (Fig 2e). Under all of these conditions CR4-uPAR proximity oscillations were observed to be in the form of a flyback sawtooth waveform of increased frequency. Therefore, several kinase inhibitors in addition to staurosporine (Kindzelskii et al., 1997) affect the frequency and waveform of CR4-uPAR oscillations.

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Oscillations were sinusoidal in both normal and SC neutrophils (neutrophils (cells (Since 0.5 µM pervanadate causes a reverse sawtooth waveform in normal cells (b), it restored sinusoidal oscillations in SC's neutrophils (e). NAD(P)H oscillations were sinusoidal in both normal and SC neutrophils (c, f).

Because the CR4-uPAR waveform of SC's cells resembled that of cells exposed to kinase inhibitors, we tested the hypothesis that inhibition of phosphatase activity could balance the diminished kinase activity of the cells, as reflected in the oscillatory waveforms. When 0.5 µM pervanadate was added to normal neutrophils a reverse sawtooth waveform was observed (the reverse of kinase inhibitors); the reduced phosphatase activity increased the declination time of the oscillatory wave (Fig 3e). The suboptimal dose of 0.5 µM pervanadate was chosen because it was previously found to only partially inhibit phosphatase activity and cell function (Bennett et al, 1993; Kindzelskii et al, 1997). When 0.5 µM pervanadate was added to SC's neutrophils, the flyback sawtooth waveform was transformed into a sinusoidal waveform (Fig 3e). Thus, inhibition of phosphatase activity balances the retarded rhythm in CR4-to-uPAR proximity to create a normal oscillatory phenotype. Unexpectedly, the period of CR4-to-uPAR proximity oscillations was increased to a normal level (20.7 ± 2.2 s; Table I), as previously associated with nonactivated neutrophils (Kindzelskii et al, 1997). Thus, the dynamical properties of inter-receptor interactions can be associated with clinical disorders.

Pervanadate restores cell polarization During these studies we noted that SC's neutrophils do not assume a triangular shape (or polarization) during in vitro spontaneous locomotion, as normal neutrophils do (Fig 4A). Furthermore, in a separate study we have shown that perturbation of intracellular oscillators into a normal sine wave pattern returns cell polarization to a normal phenotype (Adachi et al, 1998). Therefore, we tested the hypothesis that staurosporine treatment, which returns oscillations to a normal phenotype, might also affect cell shape. Inasmuch as 0.5 µM pervanadate was optimal in restoring a normal receptor oscillatory pattern, we treated SC's neutrophils under these conditions. Figure 4B shows a field of SC's neutrophils treated with 0.5 µM pervanadate at 37°C for 30 min. In comparing parts (A) and (B) of Fig 4, the number of polarized cells is substantially enhanced in the presence of 0.5 µM pervanadate. Quantitative data regarding the increase in appropriately polarized cells during spontaneous and FMLP-induced cell motility are listed in Table II. Therefore, pervanadate exposure significantly increases normal neutrophil polarization during both stimulated and unstimulated conditions. Because approximately one-half of adherent neutrophils are polarized for migration after in vitro adherence (Albrecht and Petty, 1998), pervanadate returns cell polarization to near normal levels. Thus, both dynamic receptor properties and cell functional properties associated with locomotion

Table I. Quantitative analysis of cellular oscillations in neutrophils from patient SC

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<th>No addition</th>
<th>+0.5 µM pervanadate</th>
<th>p</th>
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<tr>
<td>CR4-uPAR prox.</td>
<td>10.6 ± 1.8</td>
<td>20.7 ± 2.2</td>
<td>&lt;0.01</td>
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<tr>
<td>NAD(P)H</td>
<td>10.9 ± 2.1</td>
<td>21.1 ± 2.4</td>
<td>&lt;0.05</td>
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*Quantitative analyses of normal neutrophils were previously published (Kindzelskii et al, 1997). All experiments were repeated on at least three different days.

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Figure 2. Receptor oscillations of normal neutrophils. The RET signal intensities (ordinate) are plotted versus time (abscissa). Cells were labeled with Fab anti-CR4 and Fab’γ2 anti-uPAR reagents as previously described (Kindzelskii et al, 1997). Untreated normal migrating neutrophils exhibit a sinusoidal oscillation in CR4-uPAR RET signal (a). When incubated with 10^-7 M FMLP, a chemotactic factor, the oscillation period decreases to about 10 s (b). Incubation of normal neutrophils with suboptimal doses of kinase inhibitors perturbs the period and waveform of RET oscillations. Samples treated with 0.05 µM staurosporine (c), 150 µg genistein per ml (d), and 20 µg erbstatin per ml (e) display oscillations of ≈10 s as a flyback sawtooth waveform.

Figure 3. Receptor and metabolic oscillations of normals and pyoderma gangrenosum patient SC. Signal intensities (ordinate) are plotted versus time (abscissa). RET (a, b, d, e) and metabolic (c, f) oscillations for both normal neutrophils (a–c) and pyoderma gangrenosum neutrophils from a patient not undergoing active drug treatment (d–f) are shown. As we have reported, the RET intensity emission sinusoidally oscillates on normal cells during migration (a). Although the RET emission oscillated on cells from SC as well, a flyback sawtooth waveform was observed for these cells under untreated conditions (d). Because these results suggested reduced kinase action, we sought to “balance” this effect by reducing phosphatase activity using pervanadate. Although 0.5 µM pervanadate causes a reverse sawtooth waveform in normal cells (b), it restored sinusoidal oscillations in SC's neutrophils (e). NAD(P)H oscillations were sinusoidal in both normal and SC neutrophils (c, f).

Figure 4. Morphology of SC's neutrophils in vitro. Differential interference contrast images of SC's neutrophils are shown. Cells were viewed during spontaneous polarization in vitro at 37°C. The patient's cells do not polarize normally (A); however, inclusion of 0.5 µM pervanadate enhances cell polarization (B). Scale bar: 10 µm.
can be returned to a normal phenotype by substratifying phosphate inhibition.

**DISCUSSION**

In this and a preceding paper (Adachi et al., 1998), we have attempted to lay the groundwork for a rational understanding of pyodermangangrenosum as a dynamical disease. We have chosen to study only those cases initially presenting in childhood to increase the likelihood of obtaining cases with congenital, rather than acquired, deficiencies. Moreover, the patients are known to have in vivo dysfunctions in cell motility as evidenced by skin window tests. Because pyodermangangrenosum is a very broad and heterogenous clinical phenotype, dozens or hundreds of genotypes could account for the disorder. Thus, broad-based surveys of many patients is descriptively useful in clinical diagnoses, but of little use in sorting out cellular and molecular mechanisms. Thus, we have divided the problem into manageable units on a case-by-case basis for the present time, as the pediatric cases are relatively rare. This strategy has led to a rapid increase in our understanding of pyodermangangrenosum and presents novel ideas for rational drug intervention.

Our studies show that patient SC has a dynamic disease affecting the properties of neutrophil integrins. Although this has not been causally demonstrated, it seems likely to be of some importance given the skin window results and tissue damage. Based on insights from prior basic research (Petry and Todd, 1996), we found that the neutrophils of patient SC exhibited a kinetic CR4-to-μPAR proximity profile in the form of a flyback sawtooth waveform. This resembles the flyback sawtooth waveforms observed for normal neutrophils incubated with the kinase inhibitor staurosporine (Kindzel斯基 et al., 1997). Thus, we suggest that SC has a mutation in an integrin-associated kinase (e.g., Bohuslav et al., 1995). By treating normal cells with kinase inhibitors, it may become possible to identify the relevant integrin-associated kinases and thereby identify the aberrant gene. Although we cannot eliminate the possibility that cellular phosphate activity increased, thereby generating the observed waveform, we feel that this is unlikely because mutations almost always decrease enzyme efficiency. We have also found that the disregulated kinase-phosphate oscillatory activity can be “balanced” in this patient by the addition of pervanadate, a phosphate inhibitor. Furthermore, at this same dose of pervanadate, SC's neutrophils return to a normal polarized morphology for the present time, as the pediatric cases are relatively rare. This strategy has led to a rapid increase in our understanding of pyodermangangrenosum and presents novel ideas for rational drug intervention.

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**REFERENCES**


**Table II. Quantitative analysis of cellular polarization in neutrophils from patient SC\(^*\)**

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<tr>
<th>Condition</th>
<th>Percentage of cells polarized</th>
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<tr>
<td>Spontaneous polarization</td>
<td>18 ± 6</td>
<td>0.009</td>
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<tr>
<td>FMLP-induced polarization</td>
<td>20 ± 9</td>
<td>0.04</td>
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\(^*\)The experiments were performed on 250–500 different cells from two independent blood samples, as the patient became noncompliant.

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