Class III antiarrhythmic methanesulfonanilides inhibit leukocyte recruitment in zebrafish

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Abstract: Understanding fundamental molecular mechanisms that govern the transmigration and interstitial migration of leukocytes to sites of tissue damage and infection is of potential significance in identifying novel therapeutic targets for the management of chronic inflammatory disorders. CD31 is a mammalian cell adhesion molecule that regulates the recruitment of leukocytes from the circulation. Our recent unpublished work has suggested that homophilic ligation of CD31 can negatively regulate the ether-à-go-go-related gene (ERG) current within leukocytes to regulate cell-cell adhesion. To validate and probe the functional significance of ERG in leukocytes, we developed an infected wound model of inflammation in zebrafish and assessed the efficacy of two ERG-specific inhibitors, dofetilide and E4031, as well as an ERG-specific antisense RNA morpholino on neutrophil recruitment. Our data confirm a hitherto undescribed role for ERG in leukocytes, where inhibition or translational knockdown of ERG resulted in significant attenuation of the inflammatory response to an infectious stimulus. Inhibition of ERG was verified independently by a decrease in the ventricular heart rate, where ERG also functions in the repolarization of the cardiac action potential. Our results suggest that ERG-specific Class III antiarrhythmic drugs can modulate inflammatory responses to infection. J. Leukoc. Biol. 82: 79–84; 2007.

Key Words: inflammation · infection · ether-à-go-go-related gene (ERG)

INTRODUCTION

Zebrafish (Danio rerio) are increasingly recognized as a genetically and pharmacologically tractable organism to model and visualize the innate immune response to tissue damage and infection [1–5]. Most recently, there have been several reports that have characterized the dynamics of neutrophil recruitment to tail-fin injuries caused by tail transections [1, 3] or medial fin incisions [4]. These reports have helped define models, which will allow for rapid screening of genes that regulate leukocyte responses to injury under aseptic and septic conditions.

A key protein expressed by immune cells and essential for their recruitment from the circulation is the cell surface protein CD31, also known as PECAM-1 [6–10]. Homophilic interactions between leukocyte CD31 and endothelial CD31 are known to regulate the directed migration of leukocytes to the margin of endothelial cells in vitro [11], to promote integrin-mediated tight binding of leukocytes in vitro [12], and to regulate integrin-dependent propulsion along laminin fibers in vivo [10]. The importance of CD31 in leukocyte recruitment is best demonstrated in vivo, where antibody-blockade experiments [13, 14] or mice transgenic for a soluble plasma CD31 [14, 15] exhibit a significant attenuation of inflammatory responses. Unfortunately, the mechanism by which CD31 regulates leukocyte recruitment remains unknown.

Recently, we identified a role for CD31 in mediating leukocyte-leukocyte interactions, which allowed macrophages to discriminate between viable and apoptotic leukocytes in which a role for integrins was implicated again [16, 17]. Specifically, CD31 on live leukocytes functioned to promote disengagement and motility away from macrophages, whereas CD31 on the macrophage functioned to promote the firm binding and engulfment of apoptotic cells. In studying CD31-dependent regulation of β1-integrin binding of fibronectin-coated Latex™ beads (Fn beads) by K562 cells (a surrogate model for macrophage recognition of apoptotic cells), we discovered a role for the voltage-gated potassium channel ether-à-go-go-related gene (ERG) as a downstream effector of CD31 signaling (S. B. Brown, unpublished data).

ERG is associated more commonly with excitable cells, where it is perhaps best known as a major component of the channel responsible for the rapidly activated, delayed rectifier current (IKr) of the cardiac action potential, in which inherited mutations are associated with arrhythmias and long QT syndrome [18]. Although mammals have three distinct erg genes (erg1, erg2, erg3), zebrafish are known to possess at least four (Calum MacRae, Massachusetts General Hospital, Charles-
tum, MA, USA, personal communication), in which zerg, the equivalent of mammalian erg1, has already been reported to regulate heart rate and rhythm [19]. In nonexcitable cells, ERG has been found in cancers and cancer cell lines, particularly of epithelial and myeloid origin, where ERG is generally considered to be absent from their healthy counterparts [20]. Indeed and to the best of our knowledge, there is no prior description of ERG in primary cells of hematopoietic origin. Nevertheless, ERG1 was identified by protein cross-linking and immunocolocalization studies to associate with CD31 in K562 cells and macrophages. In this study, we sought to investigate whether zERG functioned in leukocytes to regulate a well-described, CD31-dependent process, i.e., leukocyte recruitment.

MATERIALS AND METHODS

Zebrafish husbandry and tail-fin wounding

The Golden strain was maintained according to standard protocols [21]. Embryos at 3 days postfertilization (3dpf) were anaesthetized with 20 μM tricaine (MS222, Sigma Chemical Co., St. Louis, MO, USA) before making a small wound with a 19-gauge needle to the ventral median fin posterior to the cloaca. Except for time-lapse video microscopy, embryos were removed from anesthesia after wounding and placed in fresh system water, which may have contained antiarrhythmic drugs at concentrations defined elsewhere. Although tail-fin injury was performed at room temperature, embryos were otherwise maintained at 28°C. To elicit a more pronounced leukocyte response, a Hamamatsu ORCA-ER charged-coupled device camera using a Zeiss Axioskop II MOT compound microscope, temperature controlled at 28°C with a Bioptechs Delta T open dish system. Images were typically taken at 1-min intervals for 3–4 h. Leukocyte migration was traced directly from a computer monitor before scanning into Imagine (http://rsb.info.nih.gov/ij/) from which path lengths and average migration velocities (total distance traveled/time taken) of infiltrating leukocytes were computed.

Ventricular heart rate was counted manually by two observers with the aid of a dissection microscope. Consistent with a previous report [23], no adverse effects were observed following the administration of tricaine.

Data analysis

All data were analyzed by Single Factor Anova (Microsoft Excel) with P values determined by Tukey’s post-hoc analysis or Student’s t-test. All errors are expressed as a 95% confidence interval (c.i.) unless stated otherwise. Data were plotted using Spotfire DecisionSite (www.spotfire.com).

RESULTS

Time lapse DIC microscopy of 3dpf zebrafish embryos, maintained under anesthesia, revealed an immediate contraction and distortion of the fin within the vicinity of the incision (Fig. 1, A and B, and Supplemental Movie 1). The contractive process typically resulted in a “crowning” of damaged cells.

Fig. 1. Contraction precedes leukocyte recruitment in a ventral median fin wound. (A) Embryos (3dpf) were anaesthetized with 20 μM tricaine (MS222, Sigma Chemical Co.) before making a small wound to the ventral median fin. Embryos were then imaged by DIC microscopy. (B) Images were typically taken at 1-min intervals, in which the first 6–10 min was characterized by a contractile process, resulting in the crowning of damaged cells. (C) Alternatively, embryos could be returned to an incubator maintained at 28°C for fixed time intervals before removing, fixing, and staining for MPO, and MPO-positive leukocytes are indicated with arrowheads. Original scale bars, 50 μm.

MPO staining

Embryos were fixed in 4% paraformaldehyde in distilled water for at least 1 h at room temperature before washing with 0.1% Tween 20 in PBS and staining for MPO with 0.5 mg/mL diaminobenzidine, 0.03% hydrogen peroxide in PBS/0.1% Tween 20. Embryos were then scored for MPO-stained leukocytes found within the ventral median fin and no more than 200 μm from the incision in which the depth and surface area of the wound were also recorded. Only those wounds ranging from 10 to 30 μm-deep and 500 to 2500 μm² in surface area were included for analysis.
within the first 10 min (Fig. 1B) before being sloughed off by 2 h to reveal a “v”-shaped wound (Fig. 1C). Contraction of the wound was invariably observed to pull against the caudal vein, in which severe, “deep” wounds (>70 μm or >60% of the median fin depth) typically resulted in a localized shut-down of the circulation, which rerouted via the intersegmental veins. In addition, contraction was observed to displace cells within the fin by as much as 50 μm, particularly for those cells within 120 μm of the incision (Fig. 1).

In the absence of an infectious agent, the number of infiltrating leukocytes to the site of injury was negligible, as assessed by MPO staining of fixed specimens. In contrast, the addition of a laboratory strain of P. aeruginosa (PAO-1), a natural gut microbe of zebrafish [24], to the system water (A<sub>600</sub> = 0.005) was found to elicit a leukocyte response in most (typically 60–70%) but not all embryos, which were maximal by 2 h [1.3 ± 1.2 (SD, n = 40, −PAO-1) vs. 3.6 ± 5.1 (SD, n = 357, +PAO-1)]. Analysis of DIC time-lapse imaging (Fig. 2 and Supplemental Movie 2) revealed that of 74 leukocytes, which migrated to the wound, 21 were observed to exit the ventral median fin and apparently return to circulation with a bias toward the cloaca (P < 0.14). This bias was more evident (P < 0.05) if we excluded those leukocytes, which failed to find the wound (n = 5). In contrast, leukocyte recruitment was distributed equally about the wound in which 67% of leukocytes entered the ventral median fin from the caudal vein, no further than 120 μm from the incision, the same distance that exhibited maximal distortion during the contractive phase of wound healing.

Video analysis also revealed that early transmigrating leukocytes, i.e., those transmigrating within 60 min of wounding, traveled a lengthier, “random” path to the site of inflammation, often criss-crossing their own trails before finding the wound, whereas later, transmigrating leukocytes were more likely to migrate directly from the caudal vein to the wound (Fig. 2B; P < 0.0002, Student’s t-test). Although significant variation in average migration velocities was recorded, we observed no obvious relationship with time of initial transmigration or its start-point location (Fig. 2C).

Preliminary “test” experiments of MPO-stained embryos indicated that 10 μM dofetilide in the system water was the most effective concentration at inhibiting leukocyte recruitment within the embryos (t = 2 h, P < 0.03; data not presented), although effects with 1 μM were observed (t = 2 h, P < 0.02). We also tested E4031 at 40 μM, another Class III antiarrhythmic methanesulfonanilide specific for ERG, previously reported to affect zebrafish heart rate [19]. As controls, we used Chromanol 293B (10 μM), another Class III antiarrhythmic, and nifedipine (30 μM), a Class IV antiarrhythmic. Chromanol 293B is specific for KvLQT1 (I<sub>Kr</sub>) [25], which like ERG (I<sub>Kr</sub>), functions in repolarization of the human cardiac action potential [18], whereas nifedipine is an L-type, voltage-sensitive, calcium-channel blocker. As shown, dofetilide and E4031, but neither Chromanol 293B nor nifedipine, had a profound effect on leukocyte recruitment (Fig. 3A).

The specificity of dofetilide and E4031 was confirmed when a zERG-specific morpholino [19], a well-validated technique for short-term protein knockdown in developing zebrafish embryos [22], was found to similarly affect leukocyte recruitment (Fig. 3B). It is important that inhibition of ERG, pharmacologically or translationally, also affected ventricular heart rate (Table 1). The use of dofetilide or a ZERG-specific morpholino decreased the ventricular heart rate by 15% without any apparent visible effect on caudal vein circulation. E4031 and

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**Fig. 2.** Leukocyte migration is bidirectional and focused. (A) Time-lapse video analysis of 21 individual embryos with a tail-fin wound exposed to PAO-1 over a 3- to 4-h period revealed that 14 exhibited a leukocyte response. Points of immigration and emigration across the endothelial wall are represented by the arrowheads. Gray arrowheads represent those leukocytes seen to cross the endothelial barrier in the first 90 min, whereas black arrowheads represent those seen at later time-points. Dashed, vertical arrows represent the mean position ± SD of leukocyte entry. The average ventral median fin was 105 μm-deep, and 76% of the leukocytes migrated into the fin along a 200-μm-length of caudal vein immediately adjacent and symmetrically disposed to the wound. (B and C) Leukocyte migration was traced direct from a computer monitor before scanning into ImageJ (http://rsb.info.nih.gov/ij/), from which path lengths (B) and average migration velocities (C) of infiltrating leukocytes were computed and plotted as a function of the time they entered the ventral fin.
nifedipine, which inhibited ventricular heart rate as reported previously [19], also had no apparent effect on the circulation, suggesting blood flow was not a confounding variable (Table 1).

DISCUSSION

Our data highlight an important, new target for regulating neutrophil recruitment to sites of inflammation and are consistent with our mammalian studies, demonstrating ERG1 to be a downstream effector of CD31 signaling events (S. B. Brown, unpublished data). Although the zebrafish ortholog of CD31 has yet to be identified, our ability to validate a functional role for a specific target gene in a well-described, CD31-dependent process, i.e., leukocyte transmigration [6–15], is reassuring. Precisely how ERG regulates leukocyte recruitment is the subject of active investigation. However, it is interesting to note that ERG coassociates with β1 integrins [26], and CD31 is known to regulate β1 integrins in the interstitial migration of leukocytes across the perivascular basement membrane [10]. In addition, we and others [17, 27] have shown CD31 can regulate β1 integrin binding to fibronectin-coated surfaces [27] or Latex™ beads [17]. Thus, ERG may function in leukocytes to regulate leukocyte adhesion, an essential process in transmigration and interstitial migration.

Our data are consistent with recent reports suggesting neutrophils perform a surveillance role and readily undergo bidirectional migration to and from the wound [3–5]. Of particular note is the model of Mathias et al. [4], which our wound assay replicates, and where the dynamics of neutrophil migration (and wound contraction) is essentially identical. One significant difference, however, was our requirement for the addition of a microbe to elicit a moderate response, whereas Mathias et al. [4] observed a more robust response in the absence of any

![Fig. 3. Pharmacological inhibition or morpholino knockdown of ERG blunts leukocyte recruitment.](image)

(A) The Class III antiarhythmic drugs dofetilide (DOF) and E4031, selective for ERG inhibition (IKr), blocked leukocyte recruitment to the site of injury, whereas Chromanol 293B, which is selective for KvLQT1 (IKs), and nifedipine, selective for L-type, voltage-gated calcium channels, were ineffective. Each data point represents an individual embryo fixed, stained, and counted for the number of MPO-positive leukocytes observed within the ventral fin at 2 h after wounding and exposure to PAO-1. (B) Alternatively, embryos were taken at the one-to-four cell stage and microinjected with a zERG-specific morpholino conjugated with FITC (GeneTools LLC) or a zERG 5-bp mismatch control as described [7, 11]. After 3dpf, embryos were selected for uniform uptake of fluorescein into the body of the embryo before wounding, exposing to PAO-1 for 2 h and processing as for A.

<table>
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<tr>
<th>Pharmacology</th>
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<th>Ventricular heart rate ± 95% c.i. (beats per min)</th>
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<tr>
<td>Untreated control</td>
<td>29</td>
<td>145 ± 7</td>
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<tr>
<td>10 μM Dofetilide</td>
<td>16</td>
<td>123 ± 7</td>
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<tr>
<td>40 μM E4031</td>
<td>9</td>
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<td>10 μM Chromanol 293B</td>
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<tr>
<td>30 μM Nifedipine</td>
<td>10</td>
<td>99 ± 7</td>
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<td>zERG 5 bp mismatch (5’-GAg ATc TCC GCc GCG CAC cGG gAT-3’)</td>
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<td>146 ± 10</td>
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<tr>
<td>zERG morpholino (5’-GAC ATG TCC GCG GCG CAC CGG CAT-3’)</td>
<td>25</td>
<td>124 ± 8</td>
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apparent infectious stimuli. Although this difference may be a result of the genetics of the particular zebrafish stock used (Golden vs. AB) and where we have noted extremely poor responses with Wik (data not presented), it is also possible that environmental and husbandry factors have influenced our respective results. Other studies are harder to compare, as these involved tail transections [1, 3] or laser injury of the yolk sac [2]. Regardless, our studies highlight a need to better monitor the presence of microbes and their products within the system water from which the embryos are recovered and cultured—factors, which will affect leukocyte recruitment, and an issue, which was not addressed in previous studies. Nevertheless, it is noteworthy that the effect of E4031 and dofetilide was to reduce leukocyte recruitment to levels seen when wounding was performed in the absence of PAO-1 and therefore might indicate that zERG is more important in leukocyte responses to an infected wound than to simple wounding.

Our data could also be taken to suggest that other erg genes are functional within leukocytes. This follows from the apparent weaker efficacy of the morpholino, when compared with E4031 (P<0.1) or dofetilide (P<0.2) at reducing the number of migrating leukocytes and where the drugs would have a wider use against other zERG homologs as in mammals. However, we are mindful that the amount of morpholino used (5 ng), although sufficient to affect heart rate (Table 1), was not enough to cause an atrioventricular 2:1 block, which might suggest that zERG was not knocked-down completely [19]. Finally, our data also suggest that mechno stimulation of the caudal vein may have been responsible for initial recruitment of leukocytes but that once transmigrated, leukocytes then surveyed the tissue looking for damage or infection before releasing chemoattractants for further directed leukocyte recruitment.

In conclusion, we have shown that pharmacological or translational inhibition of zERG can inhibit leukocyte recruitment to an infected wound model of inflammation in zebrafish. It is tempting to speculate that Class III antiarrhythmic methane-sulfonanilides, which are currently prescribed for cardiac arrhythmias, may have a wider therapeutic potential for the treatment of human inflammatory disorders. These same drugs, however, also promote a lengthening of the QT interval, which can be fatal in humans [18]. Although ERG is a major component of IKr, what is not known is the relative composition of erg isoforms in cardiomyocytes versus leukocytes. Zebrafish, therefore, provide an ideal system to address this issue and to screen for ERG inhibitors, which might preferentially affect leukocyte migration without being arrhythmogenic.

ACKNOWLEDGMENTS

This work was supported by a Wellcome Trust Program grant (064487, S. B. B.). J. J. M. is a Wellcome Principal Fellow, and funding support from the Wellcome Trust CVRI and the Wellcome Trust Functional Genomics Initiative is also gratefully acknowledged. We also wish to thank John Savill for his continued support and our reviewers for their helpful comments.

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