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Gcsf-Chr19 Promotes Neutrophil Migration to Damaged Tissue through Blood Vessels in Zebrafish

Jorge A. Galdames,* Constanza Zuñiga-Traslaviña,* Ariel E. Reyes,*† and Carmen G. Feijóo*

G-CSF is an essential cytokine that regulates proliferation and differentiation of granulocytes from hematopoietic stem and progenitor cells. In mammals G-CSF has been identified as a key factor that promotes the release of neutrophils from the bone marrow into the blood circulation. In silico analysis indicates that zebrafish has two gcsf genes, gcsf-chr12 in chromosome 12 and gcsf-chr19 in chromosome 19. Gcsf-Chr12 participates in emergency myelopoiesis, but, in contrast to its mammalian orthologue, is not involved in neutrophil migration toward damaged tissue. In turn, the function of Gcsf-Chr19 has not been examined yet. In this study, we analyzed the role of Gcsf-Chr19 in regulating neutrophil migration toward the wound. Our results indicated that during the first h after caudal fin transection, neutrophils migrate from the hematopoietic tissue toward the injury, using the extracellular matrix as a substrate. Later, between 3 and 4 h postdamage, the recruitment mainly occurs through the bloodstream, and only a few neutrophils still use the extracellular matrix to migrate. During this process, the transcriptional levels of gcsf-chr19 are considerably increased, reaching a peak 1 h postdamage. The knockdown of Gcsf-chr19 indicated that the percentage of neutrophils that reach the wound decreased after the first h postinjury, suggesting that the knockdown specifically affects neutrophils that travel to the wound through blood vessels. Together, our data provide novel information about the regulation of neutrophil migration in zebrafish, positioning Gcsf-Chr19 as a key signal during the course of an inflammatory process triggered by severe damage. The Journal of Immunology, 2014, 193: 372–378.

Neutrophils are mobile cells that constitute the first line of defense in the innate immune system owing to their ability to engulf and destroy microorganisms such as bacteria, fungi, and viruses. Moreover, neutrophils have a key role in eliminating cellular debris and necrotic cells when damage occurs in the absence of pathogens, known as a sterile inflammation. Under homeostatic conditions, neutrophils are retained in the bone marrow, and only a few are released into the bloodstream. In contrast, the number of neutrophils mobilized from the bone marrow is rapidly increased in response to an inflammatory stimulus.

Several studies in mammals show that the proinflammatory cytokine G-CSF has a predominant role in regulating the output of neutrophils from the bone marrow (1, 2). Levels of G-CSF expression are very low under normal conditions, unlike what occurs during inflammatory processes in which transcript levels of G-CSF dramatically increase and subsequently stimulate the migration of neutrophils to the damaged area through blood vessels.

For zebrafish, there are two paralogues of G-CSF. One is a gene located on chromosome 12 (Gcsf-Chr12; GenBank: FM174388.1), and the other is located on chromosome 19 (Gcsf-Chr19; GenBank: EU267077.1). However, only one sequence orthologous to the G-CSFR of mice exists in zebrafish; this maps to chromosome 12 (Gcsf-Chr12; GenBank: EU267077.1). Currently, there is no information about the function of Gcsf-Chr19, and very few studies have analyzed the function of Gcsf-Chr19 and Gcsf-Chr12. The single report that analyzed the involvement of Gcsf-Chr12/GcsfR in neutrophil migration after...
mechanical damage showed that both control and GcsfR morphant larvae, which received ventral fin damage, had normal levels of neutrophil migration to the wound during the first 105 min after injury (14). One explanation for this result could be that the injury model did not stimulate neutrophil migration into the blood vessels owing to the proximity of the injury with hemopoietic tissue.

Given this uncertainty, one goal of the current study was to determine if the location or intensity of the damage, or both, are crucial for activating the Gcsl neutrophil migration pathway. Three strategies were designed for causing injury in the zebrafish model. The first two designs consisted of a mild lesion located either proximally or distally to the CHT, similar to the assay previously described by Liongue et al. (14). The third design consisted of a more intense lesion, or severe damage, in which the caudal fin was completely removed. The results indicated that for the mild proximal and distal lesions, neutrophils did not migrate to the damaged area through the bloodstream, at least during the first 3 h after damage. In contrast, severe damage induced the recruitment of neutrophils to the wound mainly through blood vessels. The transcription levels of Gcsf-Chr19 during the first 3 h after severe damage were also determined, and a peak occurred 1 h postdamage (hpd).

Finally, to determine whether Gcsf-Chr19 participates in the regulation of neutrophil output from hemopoietic tissue into the bloodstream, caudal fins were removed from embryos depleted of Gcsf-Chr19. The results indicated that in a Gcsf-Chr19 knockdown situation, the percentage of neutrophils that reach the wound through blood vessels significantly decreases.

Materials and Methods

Zebrafish strains and maintenance

Zebrafish were maintained and raised according to standard protocols (15). The following strains of fish were used in this study: Tab5 (wild-type), Tg (mpx:GFP)114, Tg(chdlnb:lynGFP) (17), and Tg(fli1a:EGFP)18. All embryos were collected through natural spawning, staged according to Kimmel et al. (19), and raised at 28˚C in petri dishes containing an E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, 0.025% glucose) and 0.002% ampicillin. All maintenance and experimental protocols were reviewed and subsequently approved by the Animal Ethics Committees of the Universidad Andres Bello (Santiago, Chile), ensuring animal welfare.

Injury models

Prior to receiving any injury, larvae were anesthetized with 0.017% tricaine (20). For mild damage, a small cut to the dorsal or ventral fin was made using a microinjection needle at points proximal or distal to the CHT. For severe damage, the protocol described by Elks et al. (20) for caudal fin transection was followed. All injuries were performed on Tg(mpx:GFP)114 transgenic larvae.

Neutrophil quantification

Neutrophils were quantified according to the computational method described by Ellet and Lieschke (21). In this method, Tg(mpx:GFP)114 transgenic larvae were photographed, and every picture was analyzed using ImageJ software. Quantification was measured in leukocyte equivalent units, or the percentage of neutrophils present in the damaged tissue in relation to the total amount of neutrophils in the larval tail.

Knockdown experiments

The morpholino (Gene Tools) sequences used to inhibit splicing between exon 3 and intron 3 of the gcsf-chr19 gene are shown in Table I. Each embryo was injected with 5 ng of the respective morpholino at the one-cell stage. Diminution in gcsf-chr19 expression levels was tested through RT-PCR.

o-Dianisidine

Embryos and larvae were incubated in o-dianisidine (3,3′-dimethoxybenzidine) according to previously described methodology (22). This compound reacts with hemoglobin, generating an orange, oxidized product when in the presence of hydrogen peroxide. This reaction allows for the identification of mature erythrocytes. Briefly, the staining solution was prepared with 0.06 mg/ml o-dianisidine; 0.01 M sodium acetate; 0.65% H2O2; and 40% ethanol. Living embryos were incubated in this solution for 10 min and then fixed with 4% paraformaldehyde.

Results

Identification of the injury model that stimulates neutrophil migration through the bloodstream

The mechanical damage most used to study neutrophil migration to a wound is a cut on the ventral fin (14, 25). In this injury model, neutrophils migrate to the wound through the extracellular matrix. However, there are no reports that indicate if there is also migration through blood vessels. To determine if diverse types of damage promote differentiated pathways for neutrophil migration, three distinct types of injury were caused to Tg(mpx:GFP)114 transgenic embryos, which expresses GFP in neutrophils. Mild damage was caused either proximal or distal to the CHT (Fig. 1), whereas severe damage was simulated through the complete elimination of the caudal fin (Fig. 2).

Each type of damage was performed on a separate transgenic embryo group 2 d postfertilization, and the groups were monitored for the first 4 h with a time-lapse assay. We determined that at this time of development a zebrafish embryo has an average of 144 neutrophils in its whole body (data not shown). It was observed that in proximal and distal damage models, neutrophil migration from the CHT toward the wound was exclusively produced by the extracellular matrix (Fig. 1B, 1B’). In contrast, severe damage induced the recruitment of a more intense lesion, or severe damage, in which the caudal fin was completely removed (Fig. 2). Anothe r difference found between the proximal/distal and severe damage models was in the number of neutrophils at the circulatory loop near the injured area (Fig. 2B, 2B’). This activity was confirmed through the circulatory system (Fig. 2C). This activity was evident in the time-lapse assay with the sudden emergence of neutrophils at the circulatory loop near the injured area (Fig. 2B, 2B’, Supplemental Fig. 1). Another difference found between the proximal/distal and severe damage models was in the number of neutrophils migrating to the inflamed tissue. In the first two cases, a similar number of neutrophils was observed, but these had much fewer neutrophils in comparison with the severe damage model, in which a large number of neutrophils migrated to the wound. In the case of the proximal and distal damage model, the maximum number...
of neutrophils that reach the damaged tissue was 9. In contrast, for the intense damage model, we observe a maximum of 23 neutrophils at the wound (data not shown). Because these results indicated that only severe damage induced neutrophil migration through blood vessels, all of the following experiments were carried out using only this injury model.

Transcriptional levels of gcsf-chr19 and cxcl8-l1 during severe damage

To determine whether Gcsf-Chr19 is involved in the regulation of the immune response triggered after receiving severe damage, its transcriptional levels were assessed during the first 3 h after the start of the inflammatory process. Likewise, the mRNA levels of cxcl8-l1 were also measured, given its known role in the regulation of neutrophil migration to the wound (12, 14). Specifically, the transcriptional levels of these two genes were monitored by quantitative (q) PCR at 0.5, 1, 2, and 3 h after the injury was performed. As a control, qPCR was also performed at 0 hpd, that is, prior to damage. Results showed that even at 0.5 hpd, a significant increase in the mRNA levels of gcsf-chr19 had occurred. A peak was observed at 1 hpd, followed by a decrease, which reached nonsignificant values 3 hpd (Fig. 3A). The expression of cxcl8-l1 peaked at 1 hpd, and then levels fell before rising again at 3 hpd (Fig. 3B).

Effect of Gcsf-chr19 knockdown on neutrophil migration after severe damage

Because the qPCR results suggested an association between Gcsf-Chr19 and the immune response triggered after receiving severe damage, analysis was performed to ascertain if this cytokine controls neutrophil migration, as has been reported in mammals. A fail-of-function assay was conducted through the injection of two different morpholinos. Tg(mpx:GFP)i114 morphant embryos showed a slight decrease (from ∼19 to ∼12) in the number of neutrophils in the CHT at 30 hpf (Fig. 4A, 4B). However, this reduction was not detected at 48 hpf. Thus, the morphant phenotype was defined as those embryos with <15 neutrophils in the CHT at 30 hpf. Morpholino efficiency was checked with RT-PCR (Fig. 4G, Table I). Results showed a clear decrease in the expression of mature gcsf-chr19 mRNA and the appearance of a new band with a larger size. This band corresponded to mature mRNA, including the third intron.

To verify that the decreased number of neutrophils present in the CHT at 30 hpf was not a side effect of delayed development in injected embryos, double transgenic embryos [Tg(mpx:GFP)i114 × Tg(cldnb:lynGFP)], which have neutrophils and a fluorescently labeled lateral line primordium, were used. The control and morphant embryos were compared with the primordium positioned in the same somite (Fig. 4A, 4B). To also exclude the possibility that the knockdown of Gcsf-Chr19 triggered a general effect on hematopoietic cells, a specific erythrocyte stain was performed with o-dianisidine (21). The results indicated that the number of erythrocytes present in control and morphant embryos was indistinguishable (Fig. 4C, 4D). Finally, given the importance of blood vessels to the current study, Tg(fli1a:EGFP) embryos, which have this tissue fluorescently labeled, were used to analyze blood vessel distribution. As in the previous result, no apparent difference was observed between the blood vessel networks of morphant and control embryos (Fig. 4E, 4F).

FIGURE 1. Proximal and distal damage models. (A) Diagram showing the location of the proximal (thin dashed lines) and distal (thick dashed lines) damage zones on the ventral and dorsal fins, respectively. Gray circles represent neutrophils in the CHT. The black line corresponds to the caudal artery, and the gray line to the caudal vein. (B and C) Location (white dashed line) of neutrophils 4 h after proximal and distal damage was performed on a Tg(mpx:GFP)i114 transgenic larva. (B′ and C′) Lines tracking neutrophil migration to the wound site. (D) Quantification of the percentage of neutrophils that migrated through the bloodstream or extracellular matrix during the first 4 h after injury. The graphic shown is the same for both types of damage, near and distant. The experiment was performed three independent times using at least six larvae in each experimental group.
Having ruled out the above explanations for the observed phenotype, further determinations were made on whether the lack of Gcsf-Chr19 modified the migration route, extracellular matrix, or blood vessels used by neutrophils to reach the damaged zone. A time-lapse analysis was carried out during the first 3 h after severe damage was performed. For the first hpd in the control group, neutrophils traveled only through the extracellular matrix, but after this period, neutrophils reached the damaged area by the bloodstream (Fig. 2C, Supplemental Fig. 1).

On the basis of these data, the number of neutrophils at the injury site was compared between control and morphant embryos at 1 and 3 hpd (Fig. 5A–D). The percentage of neutrophils present at the wound was quantified by the number of neutrophils present in the CHT relative to the total number of neutrophils (24). Results showed that after 1 hpd, the number of neutrophils present at the damaged area in morphant embryos was similar to the control condition (Fig. 6A). In contrast, at 3 hpd the number of these granulocytes significantly decreased at the wound site in morphant compared with control embryos. Moreover, at this time point the number of neutrophils present at the site of injury in morphant embryos was nearly the same as at 1 hpd (Fig. 6A).

**Effect of DPI on neutrophil migration through the bloodstream**

Previous antecedents indicate that hydrogen peroxide (H$_2$O$_2$) is the main stimulus that directs the movements of neutrophils to the site of injury (26). However, it is important to note that in this previous study, only the proximal damage model was used, whereby neutrophils migrated to the wound exclusively through the extracellular matrix. The chemical compound DPI has been used to effectively inhibit the signal sent by hydrogen peroxide (22). To determine if DPI can also disrupt the migration of neutrophils through the bloodstream, damaged larvae were incubated in DPI.
before the caudal fin was removed. The results for incubated embryos indicated that fewer neutrophils reached the wound at 1 hpd, which is complementary to the unchanged number of neutrophils observed at 3 hpd (Figs. 5E, 5F, 6B).

To supplement these results, the morphant embryos were also treated with the DPI inhibitor. According to previous results, it would be expected that at both 1 hpd and 3 hpd, the number of neutrophils at the wound should decrease. This expectation was confirmed when at 1 hpd the number of neutrophils that reached the injured site in morphant embryos incubated in DPI was similar to that obtained with only the DPI treatment. Likewise, at 3 hpd the number of neutrophils that arrived to the damaged zone in morphant embryos incubated in DPI was similar to the number obtained in embryos injected with the Gcsf-Chr19 morpholino (Figs. 5G, 5H, 6B).

Discussion
Owing to the emergence of numerous transgenic zebrafish lines with fluorescently labeled neutrophils and other immune cell types (16, 27, 28), it has been possible to study the immune response from a much more complete and comprehensive viewpoint than in other vertebrates. Much of what is known about the immune system and the interactions between the cells that compose it have been studied in vitro. Given this, it is not surprising or uncommon that results differ substantially with what happens in vivo (29). The transparency of the zebrafish embryo, in addition to the wide availability of genetic resources and its conservation with higher vertebrates, makes it an ideal organism for studying the functions of the immune system.

To analyze the output of neutrophils from the CHT toward the circulatory system, it was first necessary to establish a model in which neutrophils could be effectively detected in the blood. This detection was performed using three different types of damage, thus generating different types of immune responses. Only the severe damage model, with caudal fin removal, showed the arrival of neutrophils to the wound through the circulatory system. The arrival of neutrophils via circulation was apparent from the speed at which they reached the site of injury. Directly at the damage site, it

Table I. Primers and morpholino sequences used

<table>
<thead>
<tr>
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<th>Forward (5’–3’)</th>
<th>Reverse (5’–3’)</th>
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<td>qPCR cxcl8-11</td>
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<tr>
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MO, morpholino.
was possible to observe slow neutrophil migration through the extracellular matrix, whereas other neutrophils arrived through the caudal artery and appeared almost instantaneously at the wound site. Both of these possibilities have been mentioned before (16, 30). This apparent contradiction could be explained by assuming that as neutrophils are regulated by different signals, depending on the type of inflammatory stimuli (mechanical damage, bacterial, and so on) (31), they could also respond differently according to the intensity of the same type of damage. In turn, location does not seem to be as important as intensity. The present results showed that in both a proximal and a distal injury, neutrophils traveled through the extracellular matrix to the wound. This result may be due to both models receiving a low-intensity injury, suggesting that it would not be necessary to recruit a large number of neutrophils. To test this hypothesis on wound intensity, a new assay should be performed by generating severe damage to a location proximal and distal to the CHT.

Taken together, the present results showed that neutrophil migration to the wound through blood vessels is regulated by Gcsf-Chr19. It is important to highlight that because this work was done during embryogenesis, we cannot exclude the possibility that the function of Gcsf-Chr19 may be different in adulthood. The qPCR assays showed increased transcript levels, which peaked 1 h after injury, a time point that coincides with the beginning of neutrophil migration through the circulatory system. Similar to what happened with Gcsf-Chr19, cxcl8-l1 expression levels greatly increased 1 h after damage, a result that has been previously reported (12, 13). In mice, G-CSF was observed to act as an expression regulator of CXCR2 ligands, the promigratory cytokines CXCL1 and CXCL2 (32). In humans and zebrafish, the CXCR2 ligand is CXCL8. As the current study detected a rapid increase of cxcl8-l1 mRNA, it is hard to imagine Gcsf-Chr19 acting as a regulator of Cxcl8, at least during the initial stage of the inflammatory process. To verify the above, it is necessary to measure Cxcl8 levels in Gcsf-chr19 morphant embryos. If this regulation pathway is conserved, then variations should be detected in the transcription level of Cxcl8 after damage; a study that is currently under way. The importance of the Cxcr2R and Cxcl8 in zebrafish has been previously demonstrated in a study that showed both as being necessary for neutrophil recruitment, in the case of bacterial infection, and this study also detected an increase of G-CSF in response to the infection (11). Although both cytokines Cxcl8 and Gcsf-Chr19 trigger the migration of neutrophils, the first makes it through the interstitial tissue (33) and the second via blood vessels (34). On the other hand, antecedents indicate that the Cxcl8 contribution to neutrophil migration is to attract them to the wound by biasing cell speed according to direction and by restricting cell motility near the source of the chemokine (33). According to these data and the working hypothesis of this paper, Gcsf-Chr19 should control neutrophil output from the CHT toward the blood vessels and Cxcl8, the final migration to the wound through the extracellular matrix. Future experiments will still be required to conclusively show that Cxcl8 and Gcsf-chr19 are responsible for the directional migration of tg(mpx:GFP)-positive cells toward the injury site.

For the fail-of-function assays, the percentage of neutrophils in control embryos that reached the damaged area steadily increased from 0 to 3 h (latest time tested). Meanwhile, in morphant embryos a significant increase was not observed after the first h following injury. This result was expected because it was previously determined that neutrophils reaching the wound through the bloodstream begin to travel 1 h after receiving the injury. Neutrophils migrating during the first h after the inflammatory process started

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**FIGURE 5.** gcsf-chr19 regulates neutrophil migration in response to severe damage. Tail transection was performed in 48 hpf Tg(mpx:GFP)i114 control embryos (A, B, E, and F) or gcsf-chr19 morphant (gcsf-MO) embryos (C, D, G, and H). A set of control (E and F) and morphant (G and H) embryos were also treated with DPI. Arrows indicate neutrophils at the wound. Only the knockdown of Gcsf-Chr19 affected neutrophil migration at 3 hpd. The experiment was performed three independent times using 10 larvae in each case.

**FIGURE 6.** Quantification of neutrophil migration to the wound under different conditions. (A) Percentage of neutrophils that migrated without DPI treatment. (B) Percentage of neutrophils that migrated with DPI treatment. The percentage of neutrophils that migrated was calculated as leukocyte equivalent units at the site of damage in relation to the total number of neutrophils in the CHT. The experiment was performed three independent times using 10 larvae in each case. *p < 0.05, ****p < 0.0001. MO, morpholino.
We thank Dr. Steve Renshaw for kindly providing the zebrafish transgenic line Tg(mpx:GFP)^[14].

Disclosures

The authors have no financial conflicts of interest.

References

Video 1. Neutrophil migration to the wound in a severe damage model. Lateral view of the tail of a 48hpf Tg(mpx:GFP) with caudal fin transection. The first neutrophils that reached the wound traveled through the extracellular matrix. Later, migration primarily occurred through the circulatory system. This can be evidenced with the sudden emergence of neutrophils at the circulatory loop near the injured area.